ROLE OF DHS IN TRANSLATION CONTROL OF ISLET β-CELL REPLICATION DURING HIGH FAT INDUCED OBESITY AND GLUCOSE INTOLERANCE

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology,
Indiana University

November 2017

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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July 12, 2017

DEDICATION

I dedicate this to my son, Luca Vincent Levasseur, I hope that this serves as a testament that you can accomplish anything that you set your mind to in life.

ACKNOWLEDGEMENTS

The work detailed in this dissertation is the product of dedication, commitment, persistence, and hard work from a group of passionate diabetes researchers. First and foremost, I am extremely grateful to my mentor, Dr. Raghu Mirmira. Raghu's expertise, understanding and generous guidance and support provides an outstanding example for me to follow as I pursue my passion as a scientist. It is because of Raghu's unshaken faith in my capabilities that this work has been completed. In addition, I would like to thank my research thesis committee, Dr. Carmella Evans-Molina, Dr. Maureen Harrington, and Dr. Janice Blum. The committee's guidance and support have been valuable in the path to completing my graduate school career. I would also like to thank Dr. Bernhard Maier for his friendship and valued scientific insight, as well as Dr. Sarah Tersey for her knowledge of animal physiology and experimental design skills. I would also like to thank Dr. Teresa Mastracci and Dr. Emily Anderson-Baucum for their helpful suggestions and expertise in delineating the translation aspect of this project. I am extremely thankful to Ms. Kara Benninger, whose expertise in animal husbandry and islet isolation was essential in many of the studies mentioned in this thesis. Additionally, I would like to thank the labs and members of the Center for Diabetes and Metabolic Diseases; the collaborative environment made my graduate studies very enjoyable. Lastly, I would like to thank my family, Dr. Juan Rodriguez Ramos, Rebeca Irizarry Diaz and my husband, Ryan Levasseur. Your love, encouragement, motivation and support was instrumental in the completion of this project.

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Insulin resistance in liver, muscle, and adipose tissue almost invariably occurs during obesity. To compensate, the insulin-producing β-cell increases insulin production by expanding cellular mass. The inability of the β-cell to fully compensate leads to hyperglycemia and ultimately type 2 diabetes. The enzyme deoxyhypusine synthase (DHS) catalyzes the spermidine-dependent posttranslational modification of Lys50 of eukaryotic translation initiation factor 5A (eIF5A) to form hypusine (Hyp). Studies have demonstrated this modification of eIF5A to contribute to cellular proliferation in cancerous cells, but its role in the physiologic proliferation of islet β-cells is unknown. I hypothesized eIF5A-Hyp to be required for the proliferation of islet β cells during the early phase of insulin resistance, allowing the β-cell to respond to the increased demand for insulin to maintain glucose homeostasis. To test this hypothesis, deletion of DHS was induced post-developmentally in β-cells by crossing *Dhs-fl/fl* mice with *MIP1-CreERT* mice, and animals were fed for 1 or 4 weeks with a 60% kcal from fat diet (HFD) or normal chow diet (NCD, 16% kcal from fat diet). NCD-fed and HFD-fed animals had normal glucose homeostasis after one week feeding, regardless of genotype. However, after 4 weeks of HFD, KO mice had significantly worse glucose intolerance compared to control mice. eIF5A-Hyp levels increased in β-cells of control animals and as expected remained low in the KO mice. β-cell proliferation was significantly increased after 1 week of HFD as measured by PCNA staining, however KO mice showed no increase. Cyclin D2 protein, but not mRNA, was increased in control animals fed a HFD; this protein increase was not observed in KO animals. Furthermore, polyribosomal profile of isolated islets of 1 week HFD-fed mice showed the *Ccnd2* mRNA bound to the

monoribosome fractions in the KO animals compared to the controls, resulting in changes of global translation. Interestingly, *Ccnd1* polyribosome to monoribosome ratio showed no changes in translation compared to *Ccnd2*. Taken together, these results suggest that DHS (and, consequently, eIF5A-Hyp) is necessary for the adaptive proliferative and functional response of β-cells during high fat diet induced obesity and glucose intolerance.

Raghavendra G Mirmira, MD, PhD, Chair

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ABBREVIATIONS

AKT ..... AKT Serine/Threonine kinase

ARX.....Aristaless-related homeobox

ATP .....Adenosine trisphosphate

ATRX.....X-linked helicase II

BIP ..... Immunoglobulin heavy-chain binding protein

CDC45 .....Cell division cycle 45

CDK ..... Cyclin dependent kinase

CDKN1A..... Cyclin dependent kinase inhibitor 1A

CHEK2 .....Checkpoint kinase 2

CHOP.....C/EBP homologous protein

DHS ..... Deoxyhypusine synthase

DOHH.....Deoxyhypusine hydroxylase

eIF2α..... eukaryotic translation initiation factor 2 α

eIF5A ..... eukaryotic translation initiation factor 5 A

ER ..... Endoplasmic reticulum

FFA ..... Free fatty acid

GC7..... N1-guanyl-1,7-diaminoheptane

GH..... Growth hormone

GLP-1.....Glucagon like peptide 1

GLUT2.....Glucose transporter 2

GLUT4.....Glucose transporter 4

GPR40/FFAR1 ..... Free fatty acid receptor 1

GTT ..... Glucose tolerance test

GSIS ..... Glucose stimulated insulin secretion

GSK3β.....Glycogen synthase kinase 3 beta

HFD.....High fat diet

IAPP.....Islet amyloid polypeptide

IGF-1.....Insulin like growth factor 1

INS2..... Insulin gene 2

IRS-2.....Insulin receptor substrate 2

ISL-1 ..... Insulin gene enhancer protein (Islet)

JNK ..... c-Jun N-terminal kinase

MAFA.....MAF BZIP Transcription Factor A

MAFB.....MAF BZIP Transcription Factor B

MAPK.....Mitogen activated protein kinase

MCM2.....Minichromosome maintenance complex component 2

MEN1 .....Menin

MODY .....Maturity onset diabetes of the youth

NEUROD1.....Neuronal differential 1

NF2 ..... Neurofibromin 2

NFAT..... Nuclear factor of activated T-cells

NFκB..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NKX2.2.....NK2 Homeobox 2

NKX6.1.....NK6 Homeobox 1

NOD ..... Non obese diabetic

PAX4.....Paired box 4

PBS..... Phosphate buffered saline

PDK1..... Pyruvate dehydrogenase kinase 1

PDX1..... Pancreatic and duodenal homeobox 1

PERK ..... PRKR-like endoplasmic reticulum kinase

PI3K ..... Phosphoinositide 3-kinase

PKB ..... Protein kinase B

PKC ..... Protein kinase C

PRB ..... Retinoblastoma protein

PRL ..... Prolactin

PTEN ..... Phosphatase and tensin homolog

ROS ..... Reactive oxygen species

RT ..... Room temperature

SERCA ..... Sarco/endoplasmic reticulum Ca^{2+}-ATPase

STAT ..... Signal transducers and activators of transcription

TGFB1 ..... Transforming growth factor beta 1

T1D ..... Type 1 diabetes

T2D ..... Type 2 diabetes

VDCC ..... Calcium voltage-gated channel

Chapter I

Introduction

I.A. Pancreas Structure and Function

The pancreas is a glandular organ comprised of different cellular subtypes whose divergent functions include the production of digestive enzymes to maintain whole-body glucose homeostasis. The cells of the pancreas are grossly contained within exocrine and endocrine tissues. The exocrine tissue accounts for roughly 90% of the total pancreatic mass and is responsible for producing and secreting digestive enzymes including trypsin, amylase, and lipase into the small intestine. The endocrine tissue of the pancreas, accounting for 1-2% of pancreatic mass, is entirely contained within discrete clusters known as the islets of Langerhans (or islets). There are approximately 3 million islets distributed throughout the healthy human pancreas and they are surrounded by the exocrine tissue. The islets are comprised of five different type of cells and each is responsible for producing specific hormones. The most abundant cell found in the islet is the β-cell, which produces insulin and amylin, and comprises about 70% of the islet in rodents (and perhaps 40-50% in humans) (1). The α-cell produces glucagon and comprises about 20-40% of the total islet. The δ-cell produces somatostatin and can be found in about 10% of the islet. The PP-cell, sometimes named γ-cell, produces pancreatic polypeptide and comprises about 5% of the pancreas. Lastly, the ε-cell can be found in 0-1% in the islet and produces ghrelin. The mouse islet consists of a core of β-cells, surrounded by a mantle containing the α-, δ-, PP/ γ-, and the ε-cells, whereas the human islet consists of β-cells interspersed with the other cell types. It has been proposed that human β-cells are sandwiched in between layers of α-cells folded and organized in an U or O shape (2). This proposed organization promotes heterologous contacts between α- and β-cells promoting α-cells stimulation on β-cell function, whereas

this pattern is missing in mice (3). It has been speculated that this major difference is one that partially explains the glucose sensitivity of the human β-cells compared to the rodent islets (4). The significance of this difference in cytoarchitecture in rodents vs. humans may account for the functional differences that have more recently been appreciated between the two species (5).

I.B. The Islet β Cell and Glucose Homeostasis

The islet β-cell is unique in its ability to synthesize and secrete insulin. Insulin is a highly-conserved 51-amino acid peptide hormone that is required for the cellular uptake of glucose into adipose and muscle tissue, and for the suppression of glucose output by the liver. Insulin and glucagon have opposing anabolic and catabolic functions that work in concert to maintain glucose homeostasis. After ingestion of a meal, an increase in blood glucose activates the β-cell to secrete and produce insulin through a pathway called glucose-stimulated insulin secretion (GSIS). Upon elevation of plasma glucose concentration, glucose enters the β-cell by facilitated diffusion through the glucose transporter type 2 (GLUT2) on the plasma membrane (6). Following its entry, glucose is phosphorylated by glucokinase to form glucose-6-phosphate. Glucokinase is the rate-limiting enzyme in glucose metabolism and, as such, functions as a glucose “sensor” and insulin secretion regulator (7). Glucose-6-phosphate is then subjected to glycolysis, which results in the generation of pyruvate in the cytoplasm. Pyruvate then is metabolized by pyruvate dehydrogenase to render acetyl CoA and pyruvate carboxylase to render oxaloacetate, both increasingly important in the maintenance of the citric acid cycle, and therefore facilitating the passage of molecules into the mitochondria. This reaction then leads to generation of adenosine triphosphate (ATP) in the respiratory chain and is accompanied by efflux of tricarboxylic acid cycle intermediates as anaplerosis. ATP production signals the ATP-sensitive K^+ channels in the β-cell to close,

resulting in membrane depolarization. This membrane depolarization opens L-type voltage-dependent Ca^{2+} channels (VDCC), followed by influx of Ca^{2+} and elevation of cytosolic free Ca^{2+} concentration. This elevation results in the rapid increase rate of insulin exocytosis (8).

Insulin has a short biologic half-life, thereby allowing for rapid alterations in glucose levels in response to physiologic demands. An insulin molecule made endogenously is degraded within an hour from its release into the bloodstream (9). To maintain glucose homeostasis, insulin binds to its plasma membrane receptor, inducing a conformational change and activating the receptor's tyrosine kinase function. This activation leads to autophosphorylation of the β subunit of the insulin receptor and subsequent phosphorylation of numerous downstream proteins, insulin receptor substrates, which result in transcriptional and post-transcriptional molecular effects (10). This cascade leads to the final anabolic process of glucose uptake and its conversion into glycogen, fat, and protein. Specifically, in the liver, insulin decreases gluconeogenesis and glycogenolysis, while in the striated muscle and adipose tissue, stimulating the storage of glucose as glycogen. Any remaining insulin is removed by endocytic processes in insulin-sensitive tissues or by reabsorption by the proximal tubular cells of the kidneys. Insulin also has an autocrine role in which it impedes the further release of insulin in the β-cell (11). Insulin suppresses glucagon production by the α-cell. In the fasted state, when glucose and therefore insulin levels are low, glucagon stimulates hepatic gluconeogenesis as well as the breakdown of glycogen stored in the liver to increase blood glucose levels (12). Together, these two hormones allow the body to constantly maintain glucose homeostasis. Under healthy conditions, fasted glucose concentrations do not go below 70 mg/dL or increase above 100 mg/dL. Furthermore, 2-hour post-prandial glucose should not rise above 140mg/dL. Failure of the β-cell to produce

significant insulin to meet demand and maintain glucose homeostasis is a common feature of both type 1 diabetes (T1D) and type 2 diabetes (T2D).

I.C. Transcriptional Regulation in β-cell Development and Function

Both the developing and the mature β-cell require numerous transcription factors and prohormone convertases to ensure transcription and production of insulin. Transcription factors play an important role in the maintenance of β-cell function in normal and disease states (13). Their role is to bind to a promoter of a target gene and regulate transcription. Transcription is the execution of communication between transcription factors and the basal transcriptional machinery (RNA pol II), culminating in the progression of RNA pol II through the coding region of the gene resulting in an hnRNA transcript. β-cell specific genes are transcribed via both a β-cell specific and ubiquitous transcription factors (14). Some of the ubiquitous transcription factors involved in insulin regulation include Sp, Creb, and Crem, (15–17), to mention a few. The insulin promoter has several cis-regulatory elements, such as A, C and E-box elements. These box elements are of importance to facilitate transcription initiation and enhance transcriptional activation of the target gene. Pancreatic and duodenal homeobox 1 (Pdx1) is a transcription factor central to both pancreas development and the maintenance of glucose homeostasis. *Pdx1* is first expressed in the early endoderm that is destined to form the pancreas, distal stomach, and duodenum (18). In the adult, expression of *Pdx1* is predominately restricted to the β-cell, although it is also expressed at low levels in the δ-cells of the islet as well as the acinar and duct cells. Pdx1 functions by binding DNA sequences containing short 5'-TAAT-3' DNA motifs in the 5' enhancer regions of target genes, and recruiting complexes that regulate the rate of transcription (19,20). Studies have shown that the absence or inactivation of Pdx1 as observed in *Pdx1-/-* mice and in humans with *PDX1* mutations cause pancreatic agenesis (21,22).

Haploinsufficiency of the gene (*Pdx1*+/- mice) results in maturity onset diabetes of the young 4 (MODY4) with impairment in glucose tolerance as a result of impaired insulin release (23). Pdx1 is also necessary in the mature pancreas for proper β-cell function, and transcriptional regulation of β-cell genes involved in insulin transcription (as *preproinsulin*) and secretion, including *preproinuslin*, *Glut2*, *Gck* (encoding glucokinase), *MafA*, *Nkx6.1*, and *Pdx1* itself (20,24). In the adult *Pdx1+/-* mouse, *Pdx1* deficiency results in altered islet morphology, decreased insulin, islet amyloid polypeptide (IAPP), and GLUT2 production, impaired GSIS, and glucose intolerance (23). *Pdx1* deficiency also leads to decreased β-cell mass, due in part to increased β-cell apoptosis via reduction of apoptosis regulator Bcl-2 (25). Pdx1 deficiency also leads to a loss of new β-cell formation/regeneration (26). Studies utilizing chromatin immunoprecipation and promoter microarray studies revealed numerous additional genes putatively regulated by Pdx1, including cell cycle, cell survival, exocytosis, and energy sensing genes (27). Combined, these studies indicate that Pdx1 is essential for β-cell development and function and suggest that increasing *Pdx1* expression may lead to preservation or enhancement of functional β-cell mass. Indeed, overexpression of *Pdx1* in mouse models of β-cell dysfunction enhances β-cell insulin content and GSIS, promotes β-cell mass, and glucose tolerance (28,29).

In addition to Pdx1, the Maf family of transcription factors has been found to play a role in β-cell function. These transcription factors are a distinct basic leucine zipper family of transcription factors that many studies have found to be important in the maintenance of β-cell functionality. MafB is found in both glucagon-producing and insulin-producing cells during development, but is restricted to α-cells soon after birth. MafB is capable of activating both the *insulin* and *glucagon* genes *in vitro*. In the absence of MafB, there is reduced number of insulin and glucagon cells throughout development, therefore affecting the number of mature β-cells (30). These data suggest

that MafB is involved in regulating gene integration to the formation of both β- and α-cells during the development of the pancreas. On the other hand, *MafA* is expressed in the mature β-cell and is an essential activator of the *insulin* gene. MafA is capable of activating the *insulin* gene in non-insulin producing cells (31). Deletion of *MafA* in mice results in β-cell dysfunction, glucose intolerance and impaired proliferation (32). In addition, *MafA* deletion results in a direct reduction of important genes such as *insulin*, *NeurodD1* and *Glut2,* further worsening the functionality of the β-cell during adult stages (33). Direct activation of *Pdx1* and *MafA* genes is facilitated by Islet-1 (Isl1), and mature β-cells with *Isl1* deletion results in reduced levels of Pdx1 and MafA, insulin content, and impaired insulin secretion (34).

A transcription factor that plays an important role in mature islet contributing in insulin secretion and glucose homeostasis is NeuroD1. NeuroD1 facilitates insulin secretion by modulating the activity of the K^+ channel/ATP complex; however, the exact mechanism still needs elucidation (35). *NeuroD1* deletion reduces *Ins1* mRNA expression by half resulting in a disruption of the islet architecture.

Nkx2.2 and Arx are two essential pancreatic transcription factors during development that, in their absence during adulthood, results in detrimental consequences. Nkx2.2 is required for the specification of many of the endocrine cell lineages, whereas Arx is required to obtain the correct ratio of the different cell lineages (36). *Nkx2.2* is first expressed in the pancreatic progenitor cells but ultimately is restricted to specific endocrine cells including α-, β-, PP-, and ε-cells. Loss of *Nkx2.2* expression results in absence of insulin-producing β-cells and decreased α- and PP-cells (37). *Arx*, however, is not expressed in β-cells, and when expressed under β-cell specific promoters it results in the loss of β- and δ-cells and an increase of α- and PP-cells (38).

I.D. β-cell Dysfunction in the Development of Diabetes

Diabetes mellitus is a state of absolute or relative insulin deficiency. There are five recognized main subtypes of diabetes: maturity of onset diabetes of the young (MODY), T1D, latent autoimmune diabetes of adulthood, T2D, and gestational diabetes. Diabetes is linked to problems in multiple physiological systems and has been associated with the increase in incidence of heart and kidney failure, neuropathy, lower limb amputations and vision loss (39). However, although the development of these diseases vary, β-cell dysfunction, de-differentiation, or death underlies each of these forms of diabetes. As such, research and therapies aimed at promoting β-cell function and survival are critical to promoting health in an increasingly diabetic population. From here, this introduction will focus on the pathogenesis of T2D.

T2D is a chronic metabolic disorder characterized by insulin resistance in peripheral tissues along with β-cell dysfunction. T2D is the most dominant form of diabetes encompassing 80-90% of all cases. Its increase in prevalence parallels a concomitant increase in obesity worldwide (40). Risk factors such as genetic susceptibility, obesity, and insufficient physical exercise contribute to the development of T2D; with obesity contributing to more than 50% of cases of T2D in the world (41). During obesity, insulin resistance in the muscle, liver, and adipose tissue increases the demand for insulin secretion from the islet β-cell to maintain glucose homeostasis. In an attempt to keep up with the demand, the β-cell will try to compensate by producing more insulin and/or increasing cell mass (Figure 1) (42). During this time, blood glucose levels remain the same while blood insulin levels become elevated. Studies have shown a 20-40% increase of β-cell mass in obese nondiabetic individuals (43,44). However, in individuals who develop T2D, this compensatory increase in β-cell mass and insulin production fails, leading to insulin insufficiency and frank hyperglycemia. Individuals with prediabetes (as diagnosed by impaired fasting glucose) have ~60% of normal β-cell

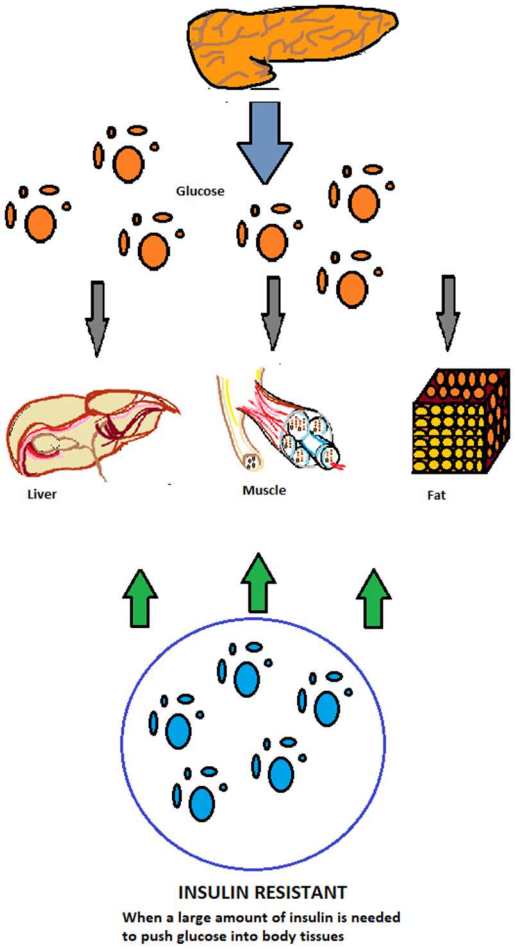


Figure 1: Insulin Resistance. Pancreatic β-cells secrete insulin to regulate blood glucose levels. Insulin triggers the uptake of glucose in the liver, muscle and adipose tissue. Diminishing insulin action on these tissues, result in blood glucose levels to increase. When insulin demand increases, the islet β-cell responds by increasing production and release of insulin to maintain glucose homeostasis.

mass and diabetic individuals have ~30-70% of normal β-cell mass (44). However, a key point is that insulin resistance alone is not an indication of future T2D, as only about 15-30% of all obese (i.e. insulin resistant) individuals develop T2D (45). This finding suggests that insulin resistance is by itself not enough to cause diabetes and therefore the inability of the β-cell to fully compensate is a major determinant in the development of dysglycemia and frank T2D (46). Curiously, humans with T2D have also been shown to have a decreased volume of pancreatic mass compared to nondiabetic individuals (44,47). Research efforts seek to understand the mechanisms that lead from insulin resistance to the progression of diabetes.

This reduction in β-cell mass in T2D resulting from increased β-cell death or inadequate replication and/or neogenesis increases the likelihood of hyperglycemia. This event places β-cells in an unfamiliar hyperglycemic environment termed glucotoxicity, which results in decreases in β-cell specific transcription factors such as Pdx1 and MafA, and decreased glucose-stimulated insulin secretion (GSIS) ultimately leading to diabetes (48). Restoring insulin exogenously early in T2D can reverse the dysfunctional insulin secretion response for at least a short period of time (49). Recent evidence points to the finding that individuals destined to develop T2D have β-cell dysfunction before signs of prediabetes (50). Diabetes research would therefore benefit from a better understanding the relationship between β-cell function, β-cell mass accrual, and loss of β-cell mass.

Circulating Mediators of β-cell Proliferation/Replication

Replication is one potential mechanism by which β-cells compensate for increased insulin demand from insulin resistance. Two fundamental concepts regarding β-cells have emerged over the past several years: 1) although β-cells were considered to be a non-replicating cell type in an adult mammal, it is now understood that they indeed exhibit a slow rate of replication in early adulthood, and replication declines rapidly as

the organism ages (51,52), and 2) the mass of β cells (and therefore the balance between new cell formation and death) can be dynamically altered to compensate for the physiologic state of the organism, as for example, following the development of insulin resistance (53). A major focus of diabetes research has been the mechanisms that underlie postnatal β-cell growth and maintenance. Multiple studies suggest that replication of β cells can be triggered or enhanced in early adulthood in rodents. Our lab and others (54–57) demonstrated that feeding of high fat diet to mice enhances β cell replication as early as 1 week following initiation of the diet.

Glucose has also been shown to promote β-cell replication in rodents. Studies in rats with continuous 20% glucose infusion for up to 96 hours resulted in a 3-fold increase in β-cell mass. These results were attributed to an increase in Akt activation, however with no increase in proliferation (58). On the other hand, studies where 50% glucose was infused for 4 days demonstrated an increase in nuclear localization and protein translation of cell cycle promoter, cyclin D2 in mouse islets, however these studies showed no differences in β-cell mass, islet number, cell size or death (55). Although an effect of glucose to directly stimulate β-cell replication has been proposed, it is possible that its effect may be caused by its stimulation of insulin release from β-cells, such that insulin in an autocrine manner, serves as the crucial mitogen. Mice lacking the insulin receptor in β-cells display impaired insulin release associated with reduced β cell mass, whereas mice lacking the IGF-1 receptor in β cells display impaired insulin release without associated loss of β cell mass (59). Interestingly, loss of both the insulin receptor and IGF-1 receptor in β cells results in severe reductions in β cell mass and frank diabetes (60). Studies ablating the intracellular signaling protein for insulin action, insulin receptor substrate 2 (IRS-2), showed a reduction in β-cell mass (61), whereas its upregulation in rat β-cells showed increased islet growth and activation of protein kinase B (Akt/PKB) (62). Notably, many studies have linked Akt expression to promote islet

growth and β-cell survival (63–65). These studies showed that overexpressing constitutively active Akt (caAkt) in the β-cell resulted in an increase in both β-cell size and islet mass (66). Other studies of mediators of PI3K signaling have supported the notion of Akt's importance in β-cell proliferation. Deletion of PDK1 (3-Phosphoinositide-dependent protein kinase 1) in mice resulted in β-cell mass reduction with insulin deficiency and deletion of PTEN (phosphatase with tensin homology) showed increased islet mass and retained cell function (67,68). Results from these and other insulin linked studies suggest that insulin plays a key role and it may not be separated from the role of glucose, suggesting both to be main players in the β-cell expansion phenomenon.

In addition to glucose and insulin, there are a large number of different secreted factors from other sources that have been evaluated for their potential critical roles in regulating β-cell mass, either by inducing replication and/or inhibiting apoptosis (69). Prolactin and growth hormone release by the pituitary gland during pregnancy also leads to β-cell stimulated proliferation. The release of these hormones is critical for maternal β-cell mass expansion during pregnancy (70). Another example is the bone-derived hormone osteocalcin, which drives β-cell replication in a cyclin D1-dependent manner (71). Further studies are warranted to determine how the β-cell is able to proliferate in the postnatal stage under certain triggers, and how this mechanism may affect turnover to alleviate elevated plasma glucose.

Lipid molecules such as free fatty acids (FFA) play many roles in cell function. For the β-cell, FFA are essential in glucose-stimulated insulin secretion, and its failure in T2D (72). Deprivation of FFA has detrimental effects on the islet; however, in chronic excess, in combination with excess glucose, FFAs can decrease insulin synthesis and secretion, and induce pathways of cellular apoptosis (73,74). It was recently discovered that FFAs are ligands to G-protein-coupled transmembrane receptors, such as GPR40/FFAR1 (75). Notably, FFAR1 is highly expressed in rodents and humans

(76,77). Studies of deficient mouse model of FFAR1 confirmed the role of the receptor in augmentation of insulin secretion, and further protection of harmful metabolic effects of HFD (78). However, in the same study, overexpression of the receptor resulted in liver steatosis, impaired islet function, and diabetes (78). As for its role in cell growth and replication, studies have suggested GPR40/FFAR1 mediates the action of oleate to promote proliferation in breast cancer cells *in vitro* (79). Its role in β-cell replication remains to be investigated.

Role of β-cell Transcription Factors in β-cell Replication

β-cell transcription factors have also been shown to play a role in β-cell replication, and may mediate, in part, the effect of the mitogens described above on β-cell replication. Perhaps the single most-important factor is Pdx1. In mouse models haploinsufficient for the gene encoding Pdx1, there is impaired β-cell mass compensation to insulin resistance, with ensuing diabetes (80). Similarly, humans with heterozygous mutations of Pdx1 develop diabetes with age, typically in adolescence or early adulthood (81,82). In these individuals, it is thought that β-cell compensation for linear growth and/or age-related insulin resistance is impaired. Pdx1 is crucial not only in the regulation of genes encoding β-cell proteins that are important in insulin secretion, such as the glucose transporter Glut2, glucokinase, and insulin, but also in the regulation of genes that are downstream of the growth-promoting insulin receptor/insulin-like growth factor 1 (IGF-1) receptor signaling cascade (83). Other β-cell transcription factors appear to play roles in β-cell replication, as well. For example, overexpression of the transcription factor Pax4 (expressed in the developing pancreas) was found to increase β-cell replication and growth in rat islets (84). Both glucose and GLP-1 increased Pax4 expression in both rats and human islets, however increased replication was not observed in the human islets (85). Another transcription factor that has recently come to

light as a possible inducer of replication is β-cell -specific homeodomain transcription factor Nkx6.1. Studies by Newgard and colleagues showed increased ^{3}H-thymidine incorporation in rat and human islets that were induced to over express Nkx6.1 (86). In addition, a number of cell cycle genes were found in rat islets to increased their expression, including cyclin-D2 (84,86).

I.E. Cell Cycle

The molecular mechanisms regulating β-cell replication are governed by cell cycle genes and their encoded proteins (87–89). Cell division consists of two processes, one in charge of DNA replication and the other one in charge of segregation of chromosome material into daughter cells. These two processes are reflected in the stages of mitosis and interphase. Cells in interphase are divided into three phases: G_1, S and G_2 (90) (Figure 2). G_1 is the interlude phase were the cell is preparing for DNA synthesis. At this stage, before committing to DNA replication, cells can enter a resting phase known as G_0. G_0 accounts for the majority of cells that are not growing and/or proliferating, and is thus the stage that reflects the majority of β-cells in an islet. Continuation of the cell cycle from G_1 is followed by the S phase, where DNA replication occurs. G_2 is the last phase in the interphase were the cell prepares for mitosis. Mitosis itself has different stages called prophase, metaphase, anaphase and telophase. Completion of cell cycle and therefore cell division occurs after all mitosis stages are completed and the end result is two daughter cells with the same genetic material. Although exceptions exist, replication of β cells is largely driven by factors that control the G1/S transition of the cell cycle (87). Genetic manipulation studies in mice have emphasized the importance of not only activators of the G1/S transition, but also of inhibitors, such that the balance between the two appear to regulate the overall drive for β-cell replication.

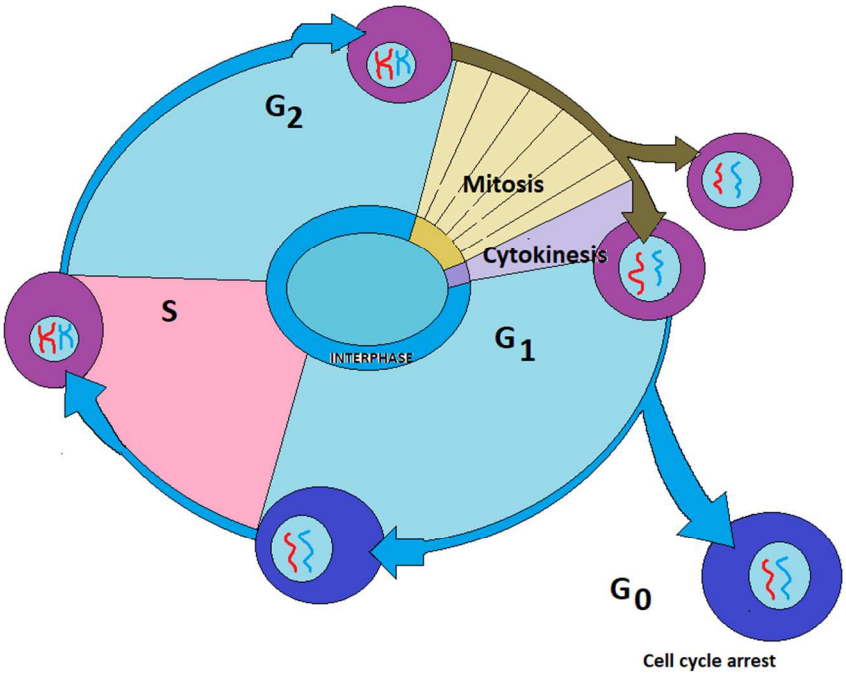


Figure 2: Cell Cycle. During interphase, the cell copies its DNA in preparation for mitosis. It is divided into 3 stages; G_1, during this stage the cell increases protein synthesis in preparation to duplicate DNA; S-phase, the cell duplicates its DNA content, and G_2, the cell resumes growth in preparation for division (mitosis). During the time the cell in active division, or preparation for division, it is in a fourth stage, G_0, were the cell is in an intermediary rest (quiescent state).

Cell cycle regulation in β-cells has recently acquired the spotlight with emerging studies implicating signaling factors and pathways to an activation in replication. As noted above, β-cell replication in humans is at its highest during infancy and declines over time, such that in by age 25-30 there are few if any replicating β-cells (51,91). These findings are paralleled in mice, where the rate of replication declines in the weeks following birth (52). The mechanisms involved in β-cell replication including their capacity to enter cell cycle are still not fully elucidated, although the precise control of replication and growth in the β-cell seem to be initiated by events involving D-type cyclins and cyclin-dependent kinases (specifically, Cdk4 and Cdk6 in mice and humans, respectively) (88). Upon mitogenic stimulation, the D-type cyclins emerge to pair with the Cdks to form complexes that allow cell cycle entry. These complexes are considered the "checkpoints" to the mitogenic stimulation. One of the first roles of these complexes is to inactivate retinoblastoma protein (pRb), which subsequently the transcription of genes that promote cell cycle progression.

The Role of D-Type Cyclins β-cell Replication

There are three D-type cyclins (1, 2, and 3), and they all respond to extra-cellular stimulation, however they each respond differently based on the stimulation and the cell type; although their function is similar if not identical in activating the cell cycle progression (92). In human pancreatic tumor samples cyclin-D1 is highly expressed (93). The general consensus is that cyclin-D1 and -D2 are the most dominant types in all cell types across different species, leaving cyclin-D3 up for debate on its presence or absence in the islet β-cell. Overall, it has been well established that cyclin-D2 and Cdk4 play an essential role in the replication of the mouse β-cell postnatally. *Ccnd1* and *Ccnd2* have been shown to increase their mRNA expression in the presence of certain hormones and growth factors. Prolactin (PRL) and growth hormone (GH) have been

shown to increase *Ccnd2* expression by activation of the Janus kinase 2 (JAK2)/Signal Transducer and Activator of Transcription 5 (STAT5) (94) (Figure 3). *Ccnd1* promoter activity is increased by incretins, GLP-1 and GIP (95). Suppression of *Ccnd1* expression has been linked to pathways such as cAMP/PKA and inhibition of phosphatidylinositol 3'-kinase (PI3K) (96). However, many other pathways have been implicated in the regulation of the D-type cyclins and their effects in β-cell proliferation. For example, *Ccnd2* expression was significantly decreased in mice with β-cell specific deletion of calcineurin b1, implicating a signaling role for calcineurin/nuclear factor of activated T cells (NFAT) in the regulation of pancreatic β-cell growth (98). Many studies of the D-type cylcins have shown that CyclinD1 and CyclinD2 are activated by distinct signals in rodents, however, this may not apply in the same fashion in human β-cells (99–101) .

Deletion of *Ccnd2* in mice resulted in a significant reduction in postnatal β-cell mass resulting in development of diabetes (102). Cyclin-D2-facilitated β-cell replication is activated via STAT5 pathway by GH and PRL. *In vitro* studies showed inhibition of GH/PRL-induced replication when a dominant negative (dn)STAT5 is transfected into the cells (94); whereas transfection with a constitutively active (ca)STAT5 showed increased replication and *CyclinD2* expression in neonatal rat β-cells and rat INS-1 insulinoma cells. Likewise, *in vivo* overexpression of dnSTAT5 in β-cells resulted in impairment of glucose tolerance in T2D model of high fat diet-induced obesity, whereas by contrast, overexpression of caSTAT5 resulted in improved glucose tolerance—due, in part, to increase in β-cell proliferation (103). Cyclin-D1 appears to have a synergistic effect in combination with cyclin-D2. Loss of the gene encoding cyclin-D1 does not affect β-cell mass accrual during embryogenesis, but heterozygosity of the cyclin-D1 gene in combination with homozygous loss of cyclin-D2 results in even further loss of β cell mass with age and severe, life-threatening diabetes (100).

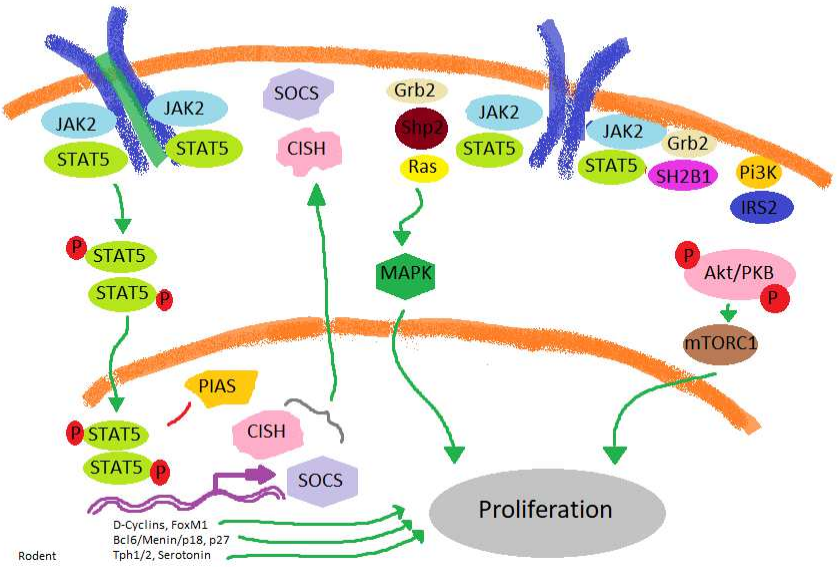


Figure 3. Activation of Proliferation. STAT activation resulting in proliferation and inhibition of apoptosis has been linked with oncogenesis. Activation of certain pathways, such as STAT5, which with its activation forms a homodimer resulting in further activation of multiple pathways with the overall goal of inducing cell growth and downregulate programmed cell death. Figure Adapted from Diabetes Article: Human β-cell Proliferation and Intracellular Signaling: Part 3 (97).

The Role of Cdks in β-cell Replication

It was suggested over a two decades ago that the D-type cyclins, in combination with Cdk4, induce proliferation specifically in the β-cell (104). Knockout of Cdk4 in mice showed developmental defects in specific organs such as ovary, testis, and pancreas; resulting in infertility and reduction of islet area. The β-cells were the only endocrine cell affected in the pancreas by whole-body *Cdk4* ablation in mice, resulting in β-cell deficiency with age (105). On the other hand, overexpression studies of *Cdk4* showed islet hyperplasia mostly populated by β-cells (105). Endogenous expression of Cdk4 rescued *Cdk4* knockout animals and restored cell proliferation and normoglycemia. Mouse isolated islets that expressed constitutively active Cdk4 and Akt showed an increase in protein expression of cyclin-D1, cyclin-D2, and p21 (a major cyclin/CDK inhibitor) (101). This induction of cyclin-D1 and cyclin-D2 coincides with an increase in phosphorylation of the downstream target of Akt, glycogen synthase kinase (GSK3β) in the islet; inactivation of GSK3β has been shown to protect β-cells against stress-induced apoptosis and instability of Pdx1 (106,107). Inhibition of GSK3 induces β-cells replication in rat islet cells and combined with high glucose and palmitate suppresses apoptosis (108).

Role of Cell cycle inhibitors in β-cell Replication

Negative regulation of the cell cycle may be as important as the mechanisms described above for positive regulation. There are two families of cell cycle inhibitors: the inhibitor of kinase 4/Alternative Reading Frame (INK4/ARF) and the CDK interacting protein/Kinase inhibitor protein (CIP/KIP). The first family, INK4/ARF consists of $p16^{INK4A}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. The second family consists of $p21^{CIP}$, $p27^{KIP}$, $p57^{KIP}$. Suppression of cell cycle inhibitors may be a key in β-cell mass maintenance, as their involvement has been implicated in islet mass changes and pathophysiology of diabetes

(109). Loss of expression of $p27^{KIP}$ in IRS-2 and LepR knockout mice leads to a massive increase in number, but not in size, of β-cells. Additionally, loss of $p27^{KIP}$ improved the hyperglycemia found in these two knockout lines (109). More recent studies have shown that the loss of $p27^{KIP}$ allows for new β-cells to proliferate. $p27^{KIP}$ knockout mice were more susceptible to development of streptozotocin-induced diabetes and had greater β-cell mass when compared to control mice. However, it seems that $p27^{KIP}$ has no influence on β-cell proliferation in neonatal stages since $p27^{KIP}$ knockout mice have to same rate of β-cell mass increase during the first three weeks after birth as wild type mice. Surprisingly, another CIP/KIP Cell cycle inhibitor, $p21^{CIP}$, has been linked to an increase in β-cell proliferation, islet mass, and insulin content that leads to hypoglycemia in mice with the hepatocyte growth factor (HGF) or placental lactogen (mPL-1) under the control of the rat *insulin* promoter. These transgenic animals had a marked increase of $p21^{CIP}$ expression. In human islets, adenoviral mediated transfection of CyclinD1 and Cdk4 resulted in upregulation of $p21^{CIP}$ and its translocation into the nucleus of the β-cell. Moreover, $p21^{CIP}$ knockout mice had no effects on islet proliferation or glucose homeostasis. The lack of $p21^{CIP}$ expression did not result in β-cell mass growth, diminishing the role of this inhibitors in cell cycle progression (110,111).

Importantly, some of cell cycle inhibitors may be expressed at different times during the lifetime of the subject. $p16^{INK4A}$ expression is widely detectable in human islet samples of adult tissues compared to youth, and it seems to be the only cell cycle inhibitor that increases expression with aging. Due to this unique feature, it has been suggested that $p16^{INK4A}$ is a biomarker for pancreatic islet aging. Overexpression of $p16^{INK4A}$ in mice resulted in a reduction of proliferation in the islet of younger mice but not in older mice. However, when β-cells deficient for $p16^{INK4A}$ were exposed to streptozotocin, they had greater rate of survival (112). These studies lead to the question of what role $p16^{INK4A}$ might have if expressed at earlier stages. It is possible that the

expression of $p16^{INK4A}$ during the aging process promotes β-cell quiescence. A quiescent cell, unlike senescent cell, can be stimulated into cell division at any given time, whereas the senescent cell is considered to be in a permanent state of growth arrest. It has been postulated in the field that the potential for β-cells to regenerate slows down in time, however, this does not mean that the cells are incapable to enter cell division under the right stimuli. It remains controversial as to what role aging may have in β-cell replication and incidence of T2D.

Tumor Suppressor Genes

Hyperplasia is the increase in volume of an organ caused by a formation of new normal cells. This phenomenon is observed primarily in embryogenesis, and in later life is primarily seen in cancer/oncogenesis (113). One gene that has been studied during growth abnormalities in parathyroid glands, anterior pituitary, and endocrine pancreas is menin. Encoded by the *Men1* gene, menin is the main cause of these abnormalities, and the disease is known as multiple endocrine neoplasia type 1 (MEN1) (114). Suppression of menin in rat INS-1 insulinoma cells results in decreased *insulin* promoter activity and decreased glucose-induced insulin secretion and proliferation (115). Heterozygous deletion of *Men1* in mice results in pancreatic hyperplasia with elevation of insulin and reduced blood glucose levels, in parallel with the human *MEN1* phenotype (116), supporting the contention that both copies of this gene are necessary for normal suppression of replication. Islets isolated from *Men1+/-* heterozygous mice have increased BrdU incorporation, consistent with hyperproliferation (117). In addition, these mice had marked mRNA and protein reductions of $p18^{INK4c}$, $p27^{KIP}$, $p15^{INK4b}$ and $p21^{CIP}$ at 10 months of age, whereas an increase of Cdk4 was found in their pancreatic islets (118). Other studies have shown that ablation of *Men1* in mice accelerated the entry of mouse embryonic fibroblasts into S phase, an increase in Cdk2 activity, and a reduction

of the cell cycle inhibitors $p18^{INK4c}$ and $p27^{KIP}$ in the pancreas (119). Furthermore, this resulted in an increase in proliferation of the islet that leads to its enlargement with no effect on the exocrine region of the pancreas. Not only does menin have a role in cell cycle molecules, but also in molecules in the apoptotic pathway. For example, caspase 8, a protein involved in the programmed cell death pathway, is transcriptionally upregulated by menin (120). Caspase 8 is induced by Fas dependent FLIP (FLICE inhibitory-protein) activity involved in cell death. Recent studies have suggested FLIP to have capability to reverse Fas signaling and induce proliferation in β-cell (119,121) These findings suggest that menin is a prime target for expanding β-cell replication and survival. Recent studies have also linked menin to gestational diabetes. During the gestational period, levels of menin, $p18^{INK4c}$, and $p27^{KIP}$ fall in the maternal islet, resulting in β-cell mass expansion (122). All these changes seem to be due to menin regulation by PRL via STAT5 signaling (123). These findings, as many others described in this section, were performed in rodents; its role in human gestation has yet to be elucidated.

Conclusions on β-cell Proliferation

Studies in the past decade trying to explain the implications in the regulation of the cell cycle during β-cell maintenance and regeneration have been performed in various mouse genetic models and confirm the complexity of factors that regulate β-cell replication. So far, we know about growth factors and mitogens and their influence on D-type cyclin expression regulation through intracellular pathways, however, how these pathways cross-talk and how β-cell replication can be influenced by autocrine signaling of insulin is still unknown. Even though knowledge has been acquired from genetic mouse models, it is imperative to consider species differences in the findings when considering therapies for diabetes in humans. For example, it has been shown that it is possible to induce regeneration of β-cells in rodents after partial pancreatectomy, duct

ligation, or HFD feeding, however this has not been observed in human adults (124,125).

I.F. Regulation of mRNA Translation in the β-cell

The rapid adjustment of protein levels in response to mitogens can be most efficiently accomplished at the level of mRNA translation. The translation of mRNA by ribosomes can be separated into three major stages: initiation, elongation, and termination (Figure 4). In the mammalian cell, distinct initiation, elongation, and termination factors are responsible for the orderly progression of these processes, and deficiency of deletion of these factors can have profound effects on global protein synthesis (126). Regulation of mRNA translation primarily occurs during either initiation or elongation phases. In eukaryotes, there are a number of different initiation and elongation factors that serve as regulators of these processes, and many of these factors can be controlled by posttranslational modifications, such as phosphorylation. Control of translation is governed by two major signaling pathways. One major signaling pathway regulating translation in response to extracellular signals and availability of amino acids includes kinases such as phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent protein kinase 1 (PDK1), Akt, and mammalian target of rapamycin (mTOR) (127). mTOR phosphorylates the inhibitory eIF4E binding proteins, which in turn release the inhibitory control over eIF4E allowing initiation to occur. In addition, mTOR facilitates translation of transcripts of elongation proteins such as eEF1A and eEF2, mRNA-binding protein PABP, and ribosomal protein S6 to increase translation capability of the cell (127). A second major mechanism for regulating translation is via the eukaryotic initiation factor 2-α (eIF2-α). Phosphorylation of eIF2-α is the result of stress sensing by kinases such as protein kinase RNA-like endoplasmic reticulum kinase (PERK), protein kinase R (PKR), general control non-derepressible 2

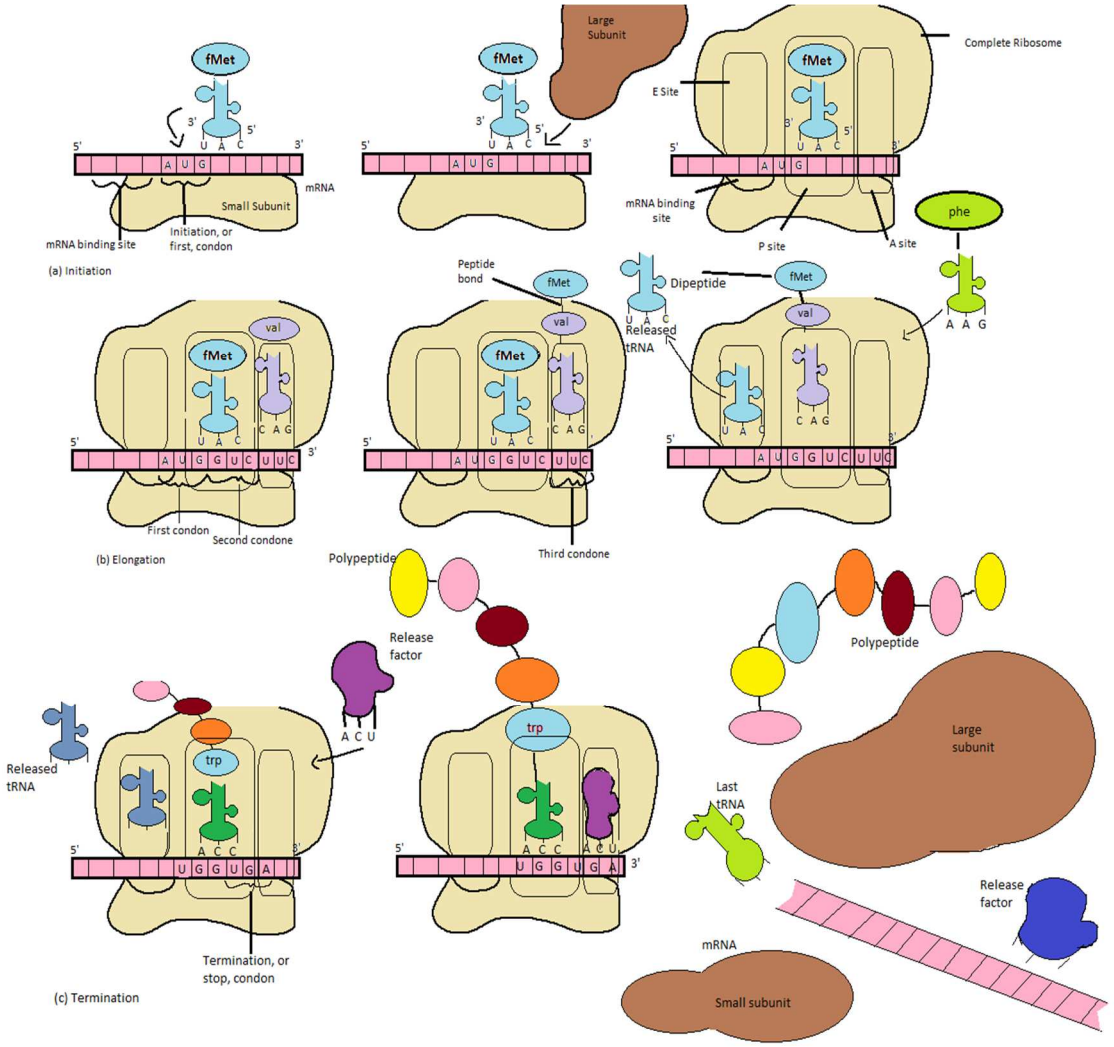


Figure 4: Mechanism of Eukaryotic Translation. Coordinated processes of initiation, elongation and termination, take place through to facilitate eukaryotic translation. This initiation process requires mRNA, ribosomal subunits, eIFs, and Met-tRNA, to initiate the process of protein synthesis at the start codon. The elongation process requires ribosome subunits, eRFs, mRNA and aminoacyl tRNAs, to polymerize the growing peptide. The termination process requires mRNA, eRFs and ribosomal subunits, to release the protein for processing and activity. Lastly ribosomal subunits and translation factors are released for recycling and to commence the process with a new transcript.

(GCN2) and heme-regulated inhibitor (HRI) (128). Each of these kinases are regulated by different intra/extracellular stress signals, including unfolded proteins in the ER lumen, viral infection, heme deficiency and amino acid starvation (129–131). All these molecules converge in the phosphorylation of residue S51 of eIF2-α, collectively known as the integrated stress response (ISR). Translation of these mRNAs is critical for mitigation of stress and activation of proliferative responses in response to extracellular signals.

The Roles of Polyamines, Deoxyhypusine Synthase (DHS), and eIF5A in mRNA Translation

Whereas most of the translation initiation and elongation factors described to date are "general" translational factors—that is, they are required for the translation of *all* mRNAs—few have been described that function to facilitate translation of only subsets of mRNAs (Figure 4). Such translational factors therefore represent prime candidates for the selective responses (e.g. proliferation) of cells to intra- and extracellular signals.

Polyamines are ubiquitous small molecules that may have potential roles in many heritable human diseases (132). The polyamines found in mammalian cells are putrescine, spermidine and spermine. These molecules form as the result of consecutive enzymatic reactions that begin with the action of arginase on arginine to form ornithine. Ornithine decarboxylase (ODC) is the rate-limiting enzyme of polyamine biosynthesis that converts ornithine to putrescine (133). The levels of ODC are dynamically and rapidly altered based on the replication state of the cells, with higher levels seen during cellular replication (134). Many physiological processes that require rapid growth such as tissue regeneration, hormonal stimulation and differentiation enhance the activity of ODC and polyamine biosynthesis. The significance of polyamines arises from their role in cellular replication, as depletion of polyamines (via inhibition of ODC) results in loss in

replication (135). Precisely how polyamines contribute to replication is not entirely clear, although it has been suggested that they function to stabilize the DNA replication fork during S phase (136). A more provocative role for polyamines, specifically spermidine, is their role in the activation of the translational factor eukaryotic translation initiation factor 5A (eIF5A).

eIF5A is a small acidic protein (~17kDa), that is highly conserved from archaebacteria to mammals, and is essential for cell viability (137). eIF5A was initially identified as a translation *initiation* factor due to its ability to stimulate methionyl-puromycin synthesis, although since its naming it is thought to function primarily as a translational *elongation* factor (138). Depletion of eIF5A in yeast results in a 30% decrease in overall protein synthesis rate, accumulation of "stalled" polyribosomes on nascent mRNAs, and in prolonged ribosomal transit times (139). Although studies of a yeast temperature-sensitive mutant of eIF5A showed a smaller effect on protein synthesis. On the other hand, in unstressed mammalian cells, depletion of eIF5A results in only a 5% reduction of mRNA translation elongation (140). These results argue against a role for eIF5A as a general translation factor, but instead suggest a more restricted role in the translation of a subset of mRNAs. Mechanistic studies in yeast have shown that eIF5A has the capacity to bind a preferential mRNA nucleotide sequence encoding consecutive proline residues and cellular target mRNAs, and that eIF5A catalyzes read-through translation of poly-proline containing proteins (141). These studies propose eIF5A, when in the proximity of the E site of the ribosome, stimulates peptidyl-transferase activity of the ribosome facilitating translation of poor substrates such as those containing consecutive proline residues. Furthermore, in agreement with the hypothesis that eIF5A works in translation control eIF5A mutant studies demonstrated that eIF5A specifically binds to actively translating ribosomes, more specifically to the 80S ribosome (142,143). However, exactly how this binding with

ribosomes occurs and/or if it occurs via protein-RNA interactions remains largely unknown. Our lab found that in the presence of cytokines, eIF5A facilitated the translation of *Nos2* in β-cells in a stress responsive manner by directly binding to the *Nos2* mRNA in the nucleus, and helping to facilitate its movement to cytoplasm of the β-cell for translation (144).

eIF5A is unique because it is the *only* protein to undergo a posttranslational modification dependent on the polyamine pathway, called hypusination (145). Hydroxyputrescine-lysine (a.k.a "hypusine") is formed during a posttranslational reaction in which a moiety of spermidine is transferred to the amino group of Lys50 of eIF5A (146) (Figure 5). This posttranslational modification essential for eIF5A's known role in translation. Hypusination is catalyzed by the enzyme deoxyhypusine synthase (DHS). Hypusination occurs in two steps: 1) DHS transfers a 4-aminobutyl moiety from spermidine to the ε-amino group of Lys50 (K50) in mammalian cells (147) to form a deoxyhypusine intermediate, 2) deoxyhypusine hydroxylase (DHH) hydroxylates the deoxyhypusine intermediate to form the hypusine-containing mature eIF5A. In yeast, mutations in the K50 residue of eIF5A results in blockade of hypusination (148,149). Additionally, when either DHS or DHH are inhibited via small molecule inhibitors, mammalian cell growth (proliferation) is halted (150–153). DHS can be chemically inhibited by N1-Guanyl-1,7-Diaminoheptane (GC7) (154,155). DHS inhibition studies have shown to not only disrupt the initial step of eIF5A maturation but also control protein biosynthesis and cell proliferation (154). An *in vivo* approach into evaluating the absence of DHS activity, studies targeting *Dhps* gene to be deleted in progeny resulted in a lack of *Dhps*$^{-/-}$ embryos (156–158). A study conducted in our lab showed DHS deletion to be embryonic lethal (156). Similarly, mouse model deletion of eIF5A resulted in embryonic lethality (157). Studies of homozygous deletion of eIF5A in blastocysts

showed growth defects as early as embryonic day 3.5 when compared to heterozygous or wild type blastocysts (157).

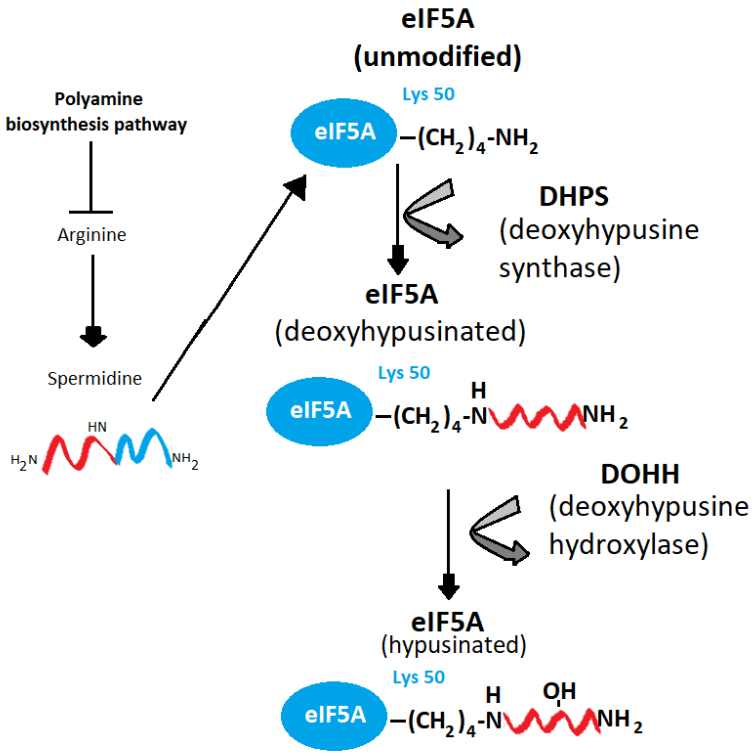


Figure 5: Hypusination of eIF5A. Two enzymes encompass the hypusination of eIF5A; deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH). These enzymes act in sequence to form a hypusine residue at the lysine 50 of eIF5A.

Recent studies have approached another mouse model to evaluate this pathway. The conditional knockout of *Dohh* (159) was also deemed embryonic lethal, and blastocyst evaluation concluded that lethality occurs before embryonic day 9.5 (159). These studies emphasize the roles of *Dhps, Dohh,* and *Eif5a* in embryonic development, but their embryonic lethality preclude an assessment of the role of these factors in cellular replication.

I.G. Conclusion and Hypothesis

Over 8.5% of the world population has T2D, and it is estimated that by 2030 it will be the 7th leading cause of death in the world (160). While a major genetic basis exists for the development of diabetes, the recent surge in prevalence underscores the influence of lifestyle, environmental, and technological factors in contributing the diagnosis and prevalence of the disease. While increasing β-cell mass by proliferation appears to have a beneficial effect in T2D, there is no direct way to only induce proliferation in a particular cell population such as the β-cell. Ideally, an increase in β-cell mass would normalize blood glucose levels, improve insulin serum concentration, or alleviate the insulin demand in a small population of β-cells. However, a therapy that induces proliferation specifically can lead to further complications such as producing tumors leading to complications harder to treat than T2D.

Based on the studies presented above and the gap in the understanding of translational mechanisms leading to compensation and proliferation in the β-cell, my dissertation research will test the hypothesis that *hypusinated eIF5A is required for the compensatory proliferation of the islet β-cell during the early phase of insulin resistance*. Furthermore, I predict that hypusinated eIF5A allows for proliferation in the β-cell by regulating the translation of cell cycle molecules important for transition between the G1/S phase. Using cell-based approaches and a conditional DHS knockout mouse

model, I tested my central hypothesis by pursuing the following aims: 1) Assess the role of β cell death and proliferation during the development of glucose intolerance in response to high fat diet (HFD) feeding in mice, 2) Assess the role of DHS in the β-cell in mice fed a normal chow diet, and 3) Delineate cellular and metabolic consequences of DHS deficiency under increased metabolic demand when mice are fed a HFD.

Chapter II

Research Design and Methods

Animal Studies. Animals were maintained under pathogen-free conditions according to protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. For Dhps knockout studies, mice containing the *Dhps* floxed alleles ($Dhps^{fl/fl}$) were crossed to previously described MIP1-CreERT mice to generate β-cell-specific Dhps knockout mice (*DhpsΔβ*) on a C57BL6 genetic background (161). Male mice were utilized for all experiments due to the gender bias found previously in many studies with Non-diabetic (NOD), Zucker Diabetic rats and others (162). In addition, it has been suggested that in some cases there are different effects of sex hormones, for example, being protective in the females as to not develop hyperglycemia in the same rate as males. Male mice with desired genotype were then weaned and utilized for all the experiments in this study. To induce genetic knockout, mice were administered three weekly intraperitoneal injections of tamoxifen (5mg) dissolved in peanut oil beginning at 8 weeks of age. One week following the final tamoxifen injection, mice were placed on either a normal chow diet (16% kcal from fat) or high fat diet (60% kcal from fat; Research Diets; D12492). Lean body mass and fat mass were obtained via dual-energy x-ray absorptiometry (DXA) using a Lunar PIXImus2 Densitometer (GE Medical Systems).

For studies involving measurement of β-cell death, male C57BL/6J mice, male C57BL/KsJ-*db/db*, and male C57BL/KsJ-*db/+* mice were obtained from the Jackson Laboratories. C57BL/6J mice were acclimated for 1 week prior to being placed on either a low fat diet (10% kcal from fat, Research Diets; D12450B) or high fat diet (60% kcal from fat; Research Diets; D12492) starting at 8 weeks of age. C57BL/KsJ-*db/db*, and C57BL/KsJ-*db/+* mice were feed a regular chow diet (Research Diets; 5008). Blood was

harvested from the tail vein and processed as serum for the differentially-methylated DNA (DMD) assay. All mice were monitored for body weight and random blood glucose via tail vein weekly. C57BL/6J mice underwent a glucose tolerance test (as described below). A subset of C57BL/6J mice from each group was euthanized biweekly and pancreata were harvested for β-cell mass measurements. After 10 weeks of diet, a subset of C57BL6/J mice received intraperitoneal injections of streptozocin (STZ) at a dose of 55 mg/kg body weight daily for 5 days.

Intraperitoneal Glucose Tolerance Test (IPGTT). Mice from all strain backgrounds were fasted overnight (~16 hours). Mice were then injected intraperitoneally with 2 g/kg body weight of D-glucose (individually-dosed) at time 0 and blood was obtained via tail collection at 0, 10, 20, 30, 60, 90, and 120 minutes post-injection of glucose. Blood glucose was analyzed using the Abbott Animal Health AlphaTRAK blood glucose meter (Abbott Laboratories, Abbott Park, IL).

Glucose-stimulated insulin secretion measurements in vivo. Mouse islets were isolated as described below. About ~50 islets per mouse were utilized to performed glucose-stimulated insulin secretion (GSIS) as described previously (163). Islets were incubated briefly in secretion assay (SAB) buffer containing 2.5 mM glucose for an hour, and then 15 mM glucose for another hour. Supernatants were collected and assayed using radioimmunometric assay for insulin for insulin (ALPCO); values were normalized to total protein content of the islet fraction.

Pancreatic Islet Isolation. Pancreatic islets were isolated from mice by collagenase digestion by the Diabetes Center Islet Core (164). Pancreata were inflated with 2.0 ml of collagenase/protease mixture (Collagenase (CIzyme MA) and Neutral

Protease (CIzyme BP)). Pancreata were then incubated at 37 °C for 15 min. Pancreata were dissociated with a series of Hank's Balanced Salt Solution (HBSS) and Bovine Serum Albumin (BSA) solutions and centrifugation and filtering steps. Islets were hand-picked, and cultured in phenol-free low-glucose Dulbecco's modified Eagle's medium prior to use. Islets were picked by hand and counted, and allowed to recover for a short period of time in 11mM glucose RPMI before experimentation.

Insulin ELISA. Upon euthanasia, blood was collected from mice by cardiac puncture in the fed state. Plasma was obtained by centrifuging blood samples at 4°C for 10 minutes at 2000 x g using a tabletop centrifuge. Plasma insulin concentrations were determined using the Mouse Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH).

DNA isolation and bisulfite conversion. DNA was extracted from mouse blood using a genomic DNA extraction kit (Sigma-Aldrich, St. Louis, Missouri). DNA was isolated from 20-50 µl of serum or cellular supernatant using QIAamp DNA Blood Mini Kit (Qiagen) with 5 µg poly-A as a carrier. DNA recovery (of the poly-A carrier) was quantified using a nanophotometer (Implen). All samples showed ≥85% recovery of DNA following isolation. Samples then underwent bisulfite conversion using the EZ DNA Methylation kit or the EZ DNA Methylation-Lightning kit (Zymo Research), and conversion was verified using a pre- and postconversion sample in the ddPCR.

Differentially-methylated DNA (DMD) assay. Primers and dual-fluorescent probes were used for interrogating methylation at CpG position -182 at the mouse Ins2 gene. The ddPCR assay was run on bisulfite-treated DNA from each sample in triplicate using a custom designed dual-fluorescent probe-based multiplex assay (AHMSN8K; Life

Technologies, Gaithersburg, Maryland). For amplification of the mouse 2 PPI promoter, the following primers were used: 5′-AATTGGTTTATTAGGTTATTAGGGTTTTTTGTTAAGATTTTA-3′ (forward); 5′-ACTAAAACTACAATTTCCAAACACTTCCCTAA-3′ (reverse). The following probes were used: 5′-CTCATTAAACGTCAACACC-3′ (VIC); 5′-CTCATTAAACATCAACACC-3′ (FAM). PCRs were performed using TaqMan® Universal PCR Master Mix, No AmpErase UNG (Life Technologies) with 60 amplification cycles at 92°C for 15 seconds followed by 60°C for 90 seconds. Water was run as a negative control for all reactions. Amplified products were subcloned into the T/A cloning vector pCR2.1 (Invitrogen, Carlsbad, California), and a minimum of 10 clones were sequenced to confirm the identity of the PCR products. DdPCR was performed using ddPCR Supermix for Probes (Bio-Rad) with the following cycling conditions: 95°C for 10 min, 94°C for 30 s, and 57.5°C for 60 s for 40 cycles. Droplets were analyzed by a QX200 Droplet Reader and QuantaSoft Software (Bio-Rad), from which a concentration (copies/μL) of methylated and unmethylated *Ins2* DNA was obtained. This final concentration was extrapolated to copy per microliter of serum and then log-transformed for parametric statistical analysis.

Morphometric assessment of β-cell mass. Pancreata from at least five different mice per group were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned onto glass slides. Pancreata from 3 mice per treatment group were rapidly dissected and weighed, fixed in 4% paraformaldehyde, paraffin-embedded, and longitudinally sectioned. Three sections per pancreas (approximately 100 μm apart) were subsequently immunostained for insulin and counterstained with hematoxylin (165), and digital images were acquired on an Axio-Observer Z1 microscope (Zeiss) fitted with an AxioCam high resolution color camera. β-cell area fraction (calculated using Axio-Vision Software) was multiplied by pancreatic weight to obtain β-cell mass. Data represent the

average from 3 sections per pancreas, and 3 pancreata from each treatment group. The β-cell mass was then calculated by multiplying the relative β-cell volume by the corrected pancreatic weight. Pancreata were immunostained for cellular proliferation/morphology changes using the following antibodies: anti-insulin (A0564, Dako, 1:1500), anti-glucagon, (PU039-UP, Biogenex, 1:100), ApopTag® Red In Situ Apoptosis Detection Kit (EMD Millipore), and nuclei were stained with Hoechst 33342, (Life Technologies).

Immunohistochemistry, immunofluorescence, and Immunoblots. Immunohistochemistry of pancreas sections were performed using anti-insulin antibodies (sc-9168 [Santa Cruz Biotechnology]; 1:250) and horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories). For immunofluorescence of pancreas sections, sections were stained with anti-hypusine eIF5A (1:100), anti-PCNA (PC10, ab29 [Abcam]; 1:200), anti-Glucagon antibody (sc-514592 [Santa Cruz Biotechnology]; 1:200), anti-DHS antibody (sc-376580 [Santa Cruz Biotechnology]; 1:200), anti-DHS antibody (ABCENT1350120 [Advanced Biochemicals (ABC)]; 1:200), and/or anti-insulin antibodies (Life Technologies; 1:250). Alexa Fluor 568–donkey anti-rabbit antibody, Alexa Fluor 488–donkey anti-guinea pig antibody and Alexa Fluor 647-donkey anti-rabbit antibody were used as secondary antibodies (Invitrogen). Images were acquired using a Panoramic MIDI (3DHistech)

Whole-cell extracts from rodent islets and MIN6 β-cells were collected in lysis buffer containing 10% glycerol, 0.1% NP-40, 0.05% deoxycholate, 50mM Tris-HCL, 10mM NaF, 1mM EDTA, and 1mM DTT and supplemented with protease and phosphatase inhibitors (Roche). Cell extracts were analyzed via immunoblot by performing separation of 15-20 μg of protein extract by 4-20% gradient SDS-PAGE (Invitrogen). Protein was transferred to a fluorescence compatible PVDF membrane

(Millipore) using a wet transfer apparatus (BioRad). Membranes were blocked with Odyssey blocking buffer (LiCor Biosciences), and incubated overnight at 4°C with primary antibodies of anti-CyclinD2 (D52F9 [Cell Signaling]), anti-DHS antibody (ABCENT1350120 [Advanced Biochemicals (ABC)], anti-actin (691001 [ImmunO]), anti-Total eIF5A(sc 18939 [Santa Cruz Biotechnology]), anti-hypusine (21st Century Biochemicals) (166), anti-PCNA (PC10, ab29 [Abcam]), anti-tubulin (sc-8035 [Santa Cruz Biotechnology]), and anti- ERK1/2 (sc-292838 [Santa Cruz Biotechnology]). Immunoblots were then washed with 0.05% Tween 20 in PBS, incubated with fluorophore labeled secondary antibodies for 1h at RT, and washed with PBS. Immunoblots were visualized and blot intensity quantified by using the LiCor Odyssey system (LiCor Biosciences).

Polyribosome Profile (PRP) analysis and RT-PCR. PRP experiments using isolated murine islets were conducted as previously described (Hatanaka et al., 2014; Templin et al., 2014). Islets were washed twice with cold PBS containing 50 µg/ml cycloheximide (CHX, Sigma-Aldrich). Islets were harvested in 500µl of lysis buffer containing 20mM Tris-HCL (pH7.5), 10mM $MgCl_2$, 100mM NaCl, 1% Triton X-100, 50U/mL recombinant ribonuclease inhibitor RNasin (Promega), and 50 µg/ml CHX. The cell lysates were passed through a 23-gauge needle and incubated on ice for 10 min, followed by centrifugation at 13,000 *x g* for 10 min at 4 °C. A portion of the lysate supernatant was preserved as the input sample to determine total mRNA levels. Supernantant (400 µl) was then added onto a 10–50% sucrose gradient solution containing 20mM Tris-HCL (pH 7.5), 5mM $MgCl_2$, 100mM NaCl, and 50 µg/ml CHX. The gradients were centrifuged at 4 °C in an ultracentrifuge at 288,000 x g for 2h (Beckman SW-41Ti rotor). A piston fractionator (BioComp Instruments, Fredericton, New

Brunswick, Canada) was used to fractionate the gradients, and absorbance of RNA at 254 nm was recorded using an in-line UV monitor (BioRad). The eluate was collected using a fraction collector, and total RNA from the fractions was reverse transcribed and subjected to quantitative RT-PCR. Polyribosome-to-monoribosome (P/M) ratios were quantitated by calculating the AUC corresponding to the polyribosome peaks (more than two ribosomes) divided by the area under the curve for the monoribosome (80S) peak. Reverse-transcribed RNA was analyzed by real-time PCR using SYBR Green I-based methodology as described in the RNA isolation below (167). The following primer sets were used for : *Cyclin D2,* 5'-GCTATGGAGCTGCTGTGCT-3' (forward) and 5'-CCAAGAAACGGTCCAGGTAA-3' (reverse) *; Cyclin D1,* 5'-GCGTACCCTGACACCAATC-3' (forward), 5'- CACAACTTCTCGGCAGTCAA-3' (reverse); Cyclin D3, 5'- GGAAGCTATGGACCAGCAAG -3' (forward), 5'-TTTGCACGCACTGGAAGTAG -3' (reverse); Cyclin A2, 5'-TCCTTGCTTTTGACTTGGCT -3' (forward), 5'- ATGACTCAGGCCAGCTCTGT -3' (reverse); *Cyclin E1,* 5'- GCTTCTAGACCTGTGCGTCC -3' (forward), 5'-CTTTCTTTGCTTGGGCTTTG -3' (reverse); Cyclin G1, 5'-AGGTCTGCGGCTTGAAACTA -3' (forward), 5'- ATTCGGATCAAATCAGTCGC -3' (reverse); CDK2, 5'- GTTGACGGGAGAAGTTGTGG -3' (forward), 5'-TGATGAGGGGAAGAGGAATG -3' (reverse); CDK4, 5'- TATGAACCCGTGGCTGAAAT -3' (forward), 5'- CCTTGATGTCCCGATCAGTT -3' (reverse); CDK6, 5'-GCCTATGGGAAGGTGTTCAA -3' (forward), 5'- GGGCTCTGGAACTTTATCCA -3' (reverse); p18, , 5'- AATGGATTTGGGAGAACTGC -3' (forward), 5'-TGACAGCAAAACCAGTTCCA -3' (reverse); p21, 5'- TCCAGACATTCAGAGCCACA -3' (forward), 5'- GACCCAGGGCTCAGGTAGA -3' (reverse); p27, 5'-GATACGAGTGGCAGGAGGTG -3' (forward), 5'- TTCTGTTCTGTTGGCCCTTT -3' (reverse); p57, 5'- CACTCTGTACCATGTGCAAGGAGTA -3' (forward), 5'-

TTTCTCTTTTTGTTTTGCACTGAGA -3' (reverse); Menin, 5'-TTCAGCTTCATCACAGGCAC -3' (forward), 5'- ACCACCCAAGCATGATCTTC -3' (reverse); Ki67, 5'- CTGCCTGCGAAGAGAGCATC -3' (forward), 5'- AGCTCCACTTCGCCTTTTGG -3' (reverse); PCNA, 5'- ACCTGCAGAGCATGGACTCG -3' (forward), 5'- GCAGCGGTATGTGTCGAAGC -3' (reverse); INS1, 5'- ACCTTTGTGGTCCTCACCTG -3' (forward), 5'- AGCTCCAGTTGTGGCACTTG -3' (reverse); INS2, 5'- TGTGGTTCTCACTTGGTGGA -3' (forward), 5'- CTCCAGTTGTGCCACTTGTG -3' (reverse); PDX1, 5'- CTCCGGACATCTCCCCATAC -3' (forward), 5'- ACGGGTCCTCTTGTTTTCCT -3' (reverse); GK, 5'- AAACTACCCCTGGGCTTCAC -3' (forward), 5'- CCACGATGTTGTTCCCTTCT -3' (reverse); GLUT2, 5'- GGCACAGACACCCCACTTAC -3' (forward), 5'- GCCAACATTGCTTTGATCCT -3' (reverse); and Actin, 5'- AGCCATGTACGTAGCCATCC -3' (forward), 5'- CTCTCAGCTGTGGTGGTGAA -3' (reverse). Samples were reported as the percent of total recovered RNA. Samples utilized for real-time RT-PCR had approximately 10^6 cells or 50-100 islets isolated in 350 ml of Buffer RLT (Qiagen) containing 1% β-mercaptoethanol. Samples were then sheared through a 27-gauge needle, and RNA isolation was processed as described below. All data represent the mean of triplicate determinations from at least three independent experiments of MIN6 cells or pooled mouse islets from three separate isolations.

RNA isolation and analyses. MIN6 β-cells and isolated murine islets were lysed using Qiagen Buffer RLT with 1% β-Mercaptoethanol and RNA was isolated using an RNeasy mini kit (Qiagen). Purified RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reactions were performed in a 96-well plate using the Mastercycler Nexus Gradient (Eppendorf). Each reaction

contained the following: 0.75 µl of SYBR GREEN (Applied Biosystems), 10 pmol/µl Primer mix, 3 µL of cDNA, and RNase free water to a total volume of 25 µl. The PCR conditions used were 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 40 s. Cycle threshold values were obtained and normalized to *Actin*. The ΔΔCt method was used to determine relative expression levels (168). RNA from isolated islets was sent to the Center for Medical Genomics Core in IU School of Medicine for RNA deep-sequencing analysis.

Cell Isolation and Culture. MIN6 β-cells were maintained in 25 mM glucose. Mouse islets were cultured in 11 mM glucose and were allowed to recover for one hour prior to experimentation. On the evening prior to experimentation, cells were incubated in medium containing 5.5 mM glucose, and on the morning of experimentation, glucose concentration was increased to 20 mM. For PRP experiments, islets were transferred into cold RPMI medium containing 5 mM glucose after isolation and used immediately. Sodium palmitate, sodium oleate, and fatty acid–free BSA, were purchased from Sigma-Aldrich. Free fatty acid (FFA) and fatty acid–free BSA conjugates (at an 8:1 molar ratio of FFA to BSA, unless otherwise stated) were prepared as described previously (Listenberger et al., 2001). Control cells were treated identically in terms of glucose concentrations, except that BSA was added without FFA. Protein samples were then submitted for Reverse Phase Protein Array (RPPA) at the University of Texas Anderson Center for profiling.

RNA Immunoprecipitation (RIP). RIP assays from 1×10^7 formaldehyde cross-linked MIN6 cells were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMDMillipore). All steps in the protocol were followed with the

exception of reversal of cross-linking, which required a 2-hour incubation at 65 ºC. Isotype-matched antibodies against eIF5A and the FLAG-Ctl epitope (for control immunoprecipitations) were used at a final dilution of 1:100. Immunoprecipitated RNA was reverse-transcribed and subjected to real-time PCR amplification for selected genes, as described above. All data represent the average of triplicate determinations from at least 3 independent RIP assays.

Statistical Analysis. All data are presented as mean ±SEM. For comparisons of methylated and unmethylated mouse *Ins2* DNA levels, a two-tailed unpaired Student's t test was used. For analysis of methylated and unmethylated *INS* DNA levels, a Kruskal-Wallis (non-parametric) test was employed followed by a Dunnett's post-test (to compare values to healthy controls). Statistical significance was assumed at $P<0.05$.

Chapter III

Transient β-cell Death during Obesity and Glucose Intolerance

In this chapter, I explore the possibility of β-cell death under high fat diet feeding in mice by measuring a circulating biomarker. Experiments with mice were carried out in collaboration with Eli Lilly Co. scientists in the Diabetes department.

III.A. Introduction

The demand on insulin production and secretion is increased in the presence peripheral-tissue insulin resistance, as seen in the setting of high fat diets (HFDs) (169). During HFD feeding, a major factor in the progression from normoglycemia to hyperglycemia is the inability of the β-cell to fully compensate for peripheral tissue insulin demands (170–173). At least two mechanisms exist to account for insufficient insulin secretion: reduction in β-cell function or a reduction in β-cell mass (or both). Islets isolated from humans and rodents with type 2 diabetes (T2D) suggest that β-cell dysfunction (as measured by insulin release in response to a glucose challenge of islets in vitro) occurs (174–176). Evidence for reduced β-cell mass also exists. For example, cross-sectional studies in humans using autopsy specimens have shown that β-cell mass is reduced in individuals with type 2 diabetes (T2D) compared to obese or lean non-diabetic individuals (177,178). Together, these findings suggest that a combination of reduced function and mass of β-cells contribute to the observed lack of insulin secretory capacity in T2D.

Genome-wide associated studies support the idea of variants in genes that regulate β-cell function and development contribute to the risk of β-cell dysfunction in the setting of T2D (179). However, the precise mechanism(s) accounting for the reduction in β-cell mass in T2D remain elusive. Because studies of β-cell mass in humans emanate

from cross-sectional autopsy specimens, it remains possible that the observed reduction in β-cell mass pre-existed in these individuals and did not reflect a dynamic change. Nevertheless, a working hypothesis in the field is that dynamic loss of β-cells via death or de-differentiation accounts for the progression to T2D. Cadaveric donor studies have shown that β-cell loss could be the result of β-cell apoptosis (177), whereas other studies have suggested dedifferentiation as a key mechanism for β-cell loss in mice and humans (180,181).

Our lab and other groups have established an assay that measures circulating unmethylated DNA encoding preproinsulin as a biomarker of β-cell death (182–184). β-cells have a higher frequency of unmethylated CpG residues in the DNA encoding preproinsulin compared to other cell types (185). Upon death, the β-cell releases its DNA into the circulation, and its detection in the circulation is evidence of β-cell death. The differentially-methylated DNA (DMD) assay is the technique proposed as a method of measuring β-cell death *in vivo* (184,186–189). The DMD assay, performed on bisulfite-treated DNA (to convert unmethylated cytosines to uracils), utilizes a single-nucleotide polymorphism discrimination PCR-based technique (TaqMan® probes) to detect the presence or absence of cytosine methylation at a given CpG site (182,184). In this respect, the assay discriminates species of DNA liberated from dying β-cells (unmethylated CpG) and DNA liberated from dying non- β-cells (methylated CpG).

Multiple different DMD assays to detect β-cell death have been described (182–184,189,190). The assays differ with respect to the particular CpG site being detected and the technique by which it is measured. The assay developed and utilized by our group assesses the methylation status of the CpG site at position -182 bp relative to the start site of the mouse *Ins2* gene (184). The assay detects β-cell death in the circulation in mice following streptozotocin (STZ) treatment and prior to the occurrence of diabetes in type 1 diabetic non-obese diabetic (NOD) mice (184). A limitation of these prior

studies was that real-time PCR provided only *relative* quantitation of DNA; instead, knowing absolute levels of DNA (as in copy numbers per unit volume) might allow for more precise and sensitivity determination of the magnitude of β-cell loss. Recently, our group and others have employed digital PCR (ddPCR), a technique that provides absolute quantitation of DNA fragments, requires only microliter quantities of serum from fresh or banked samples, and shows greater sensitivity than traditional quantitative PCR (191,192). DdPCR technology involves the use of conventional TaqMan® assays and a microfluidics-based partitioning of a PCR reaction into 10K-20K droplets. Following thermal cycling of the partitioned reaction, the droplets are analyzed by a flow cytometer to identify those with positive and negative signals. Poisson statistics is used to calculate absolute quantities (copy numbers) of each DNA species (193). The DMD assay coupled to ddPCR technology has yet to be applied to assess β-cell death in the T2D disease progression. The studies in this chapter utilize the DMD assay (with dPCR) in *C57BL/6J* mice fed a HFD compared to control mice on a low-fat diet (LFD), and in diabetic *C57BLKs/J-db/db* mice compared to control *db/+* mice. My results suggest that β-cell death occurs in an episodic, rather than continuous, fashion during the course of obesity.

III.B. Results

β-cell death DNA assay development.

To assess β-cell death in mouse models of obesity and T2D, I modified our established real-time PCR-based DMD assay (184) for compatibility with the more sensitive and specific ddPCR technique that allows for absolute quantitation of DNA copy numbers. The primers described in that study interrogated differential methylation of cytosine at position –182 bp (relative to the transcriptional start site) of the mouse *Ins2* gene. I validated the specificity of these primers in ddPCR using plasmids containing

cloned methylated or unmethylated *Ins2* gene. As shown in the 2-dimensional ddPCR plots in Figure 6A, the primers quantitatively distinguished mixtures of these plasmids. Figure 6B-D shows that the primers linearly and quantitatively detected mouse islet DNA spiked into mouse serum. I then applied this DMD assay to a mouse model of obesity and impaired glucose tolerance (IGT) and to a mouse model of T2D, both followed longitudinally.

Glucose intolerance in mice after HFD feeding for several weeks

To determine how obesity affects glucose homeostasis, β-cell compensation, and β-cell death, I treated male *C57BL/6J* mice with either a high fat diet (HFD; 60% kcal from fat, 20% from protein, 20% from carbohydrate, Research Diets D12492) or a control, low fat diet (LFD; 10% kcal from fat) starting at 8 weeks of age. *C57BL/6J* HFD-fed mice exhibited statistically worse glucose tolerance starting as early as 2 weeks after the start HFD feeding and was worse at 6 and 10 weeks after the start of HFD feeding (Figure 7A-C). As expected, mice fed a HFD had a significant increase in percent body fat compared to LFD-fed mice by dual-energy X-ray absorptiometry (DXA), and body fat increased over the timeframe of the study (Figure 7D). Taken together, these results establish that the HFD feeding paradigm leads to obesity and impaired glucose tolerance (IGT) over time.

C57BL/6J HFD-fed mice exhibited statistically increased body weights and fasting blood glucose values compared to control LFD-fed animals beginning at 6 weeks after starting the diet (Fig. 8A and B). We assessed glucose tolerance in HFD-fed mice on a weekly basis; as shown in Fig. 8C, glucose tolerance was statistically worse at 2 weeks of age and continued through the end of the study. β-cell mass, as quantified histologically. increased significantly in HFD-fed animals compared to controls by 6 weeks post diet initiation (Fig. 8D). Compared to LFD-fed control animals, HFD-fed mice

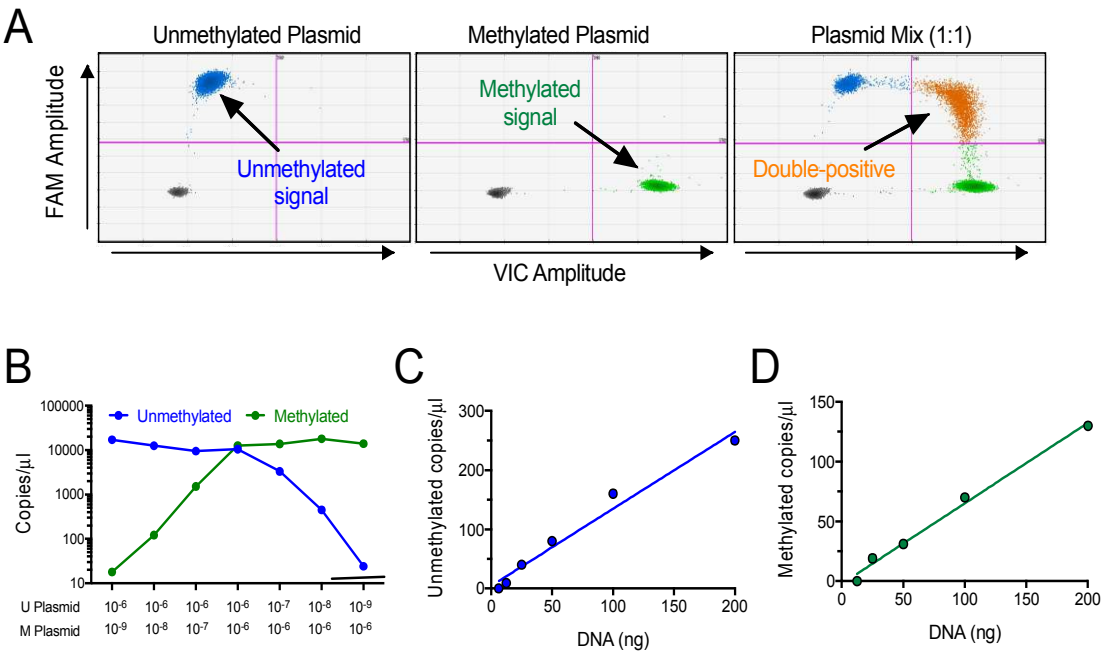


Figure 6: Mouse DMD assay specificity and validation. (**A**) Two-dimensional plots using plasmid standards for unmethylated and methylated mouse *Ins2* DNA and for a 1:1 mixture of the two plasmids. Arrows identify the unmethylated, methylated, and unmethylated + methylated (double-positive) *Ins2* DNA-containing droplets. (**B**) Quantitation of plasmid dilution curves, presented as copies/µl. (**C, D**) Quantitation of dilution curves of serum spiked with mouse DNA for (C) unmethylated *Ins2* DNA and (D) methylated *Ins2* DNA. R^2 = 0.9733 for unmethylated *Ins2* DNA and R^2 = 0.9917 for methylated *Ins2* DNA.

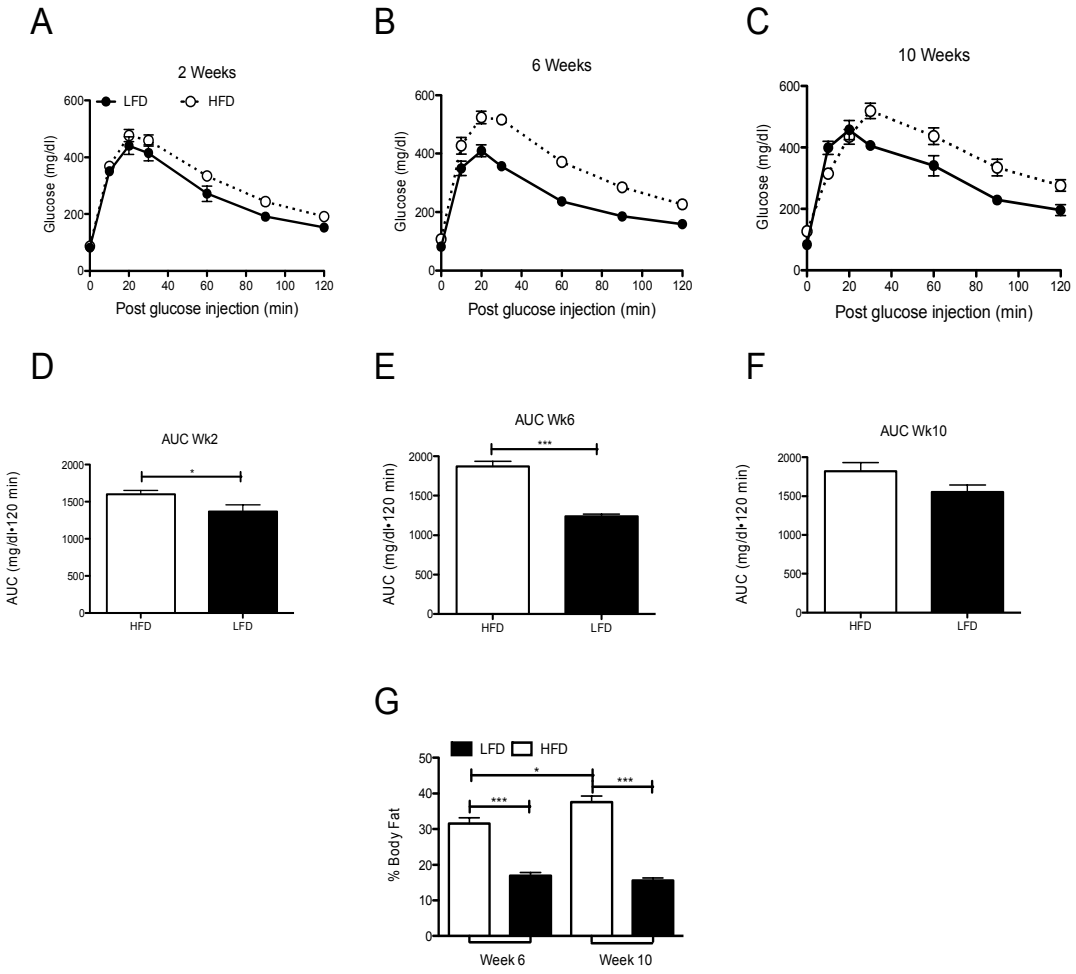


Figure 7: Glucose Intolerance during LFD and HFD feeding in C57BL/6J. Mice were fed a LFD or HFD starting at 8 weeks of age. Every 2 weeks, a cohort of mice in each group was evaluated for glucose intolerance, and body fat content to determine correlation with β-cell death. (**A**) Glucose Tolerance Test after 2 weeks on diet; (**B**) Glucose Tolerance Test after 6 weeks on diet; (**C**) Glucose Tolerance Test after 10 weeks on diet; (**D**) Area under the Curve (AUC) of GTT at 2 weeks of diet; (**E**) Area under the Curve (AUC) of GTT at 6 weeks of diet; (**F**) Area under the Curve (AUC) of GTT at 10 weeks of diet; (**G**) DEXA scanning after 6 and 10 weeks on diet. N=8 mice total for each of 4 groups, done on two separate occasions. Data are presented as mean ± SEM; **P*<0.05 for HFD at 6 weeks of diet compared to 10 weeks of diet, ***P<0.0001 for HFD compared LFD.

exhibited episodic increases in unmethylated *Ins2* DNA levels at 2 and 6 weeks post diet initiation (Figure 8E), coincident with the time points where glucose levels, fasting and by glucose tolerance test (GTT) were elevated (Figure 8B and C). By contrast, methylated *Ins2* DNA levels were not statistically different in HFD-fed animals compared to controls across the feeding period. At 10 weeks after starting diet, LFD-fed mice were administered multiple low doses of STZ to induce overt β-cell death. As shown in Figure 8E and F, unmethylated *Ins2* (but not methylated *Ins2*) DNA levels increased significantly 1 week following STZ injections, then declined to baseline levels. The unmethylated *Ins2* levels increased in HF-fed animals at two and six weeks of diet correlating to the first-time points demonstrating glucose intorelance and hyperglycemia. To correlate the serum β-cell death biomarker with a more traditional cell death marker, tissue sections were stained using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. As shown in Figure 8G and H, STZ-treated mice exhibited increased β-cell death as evidenced by increased numbers of TUNEL+Insulin+ cells. In Figure 8H quantification of TUNEL+Insulin+ cells were quantified in pancreata sections across the whole pancreas to determine the ratio of β-cell death under HFD compared to LF-fed animals. Although no statistically significant increases in TUNEL+Insulin+ cells were seen at 2, 4 or 6 weeks of HFD feeding, a trend toward higher numbers were observed at 2 and 6 weeks of HFD feeding, correlating to the same timepoints at which we observed an increased in unmethylated *Ins2.*

Absence of β-cell death in mice with established T2D.

Next, we applied the DMD assay to a mouse model of spontaneous T2D. The *db/db* mouse on the *C57BLK/sJ* background is an inbred strain that harbors a mutation in the leptin receptor gene and exhibits obesity, insulin resistance, β-cell dysfunction, and diabetes as early as 6 weeks of age on a normal chow diet (194).

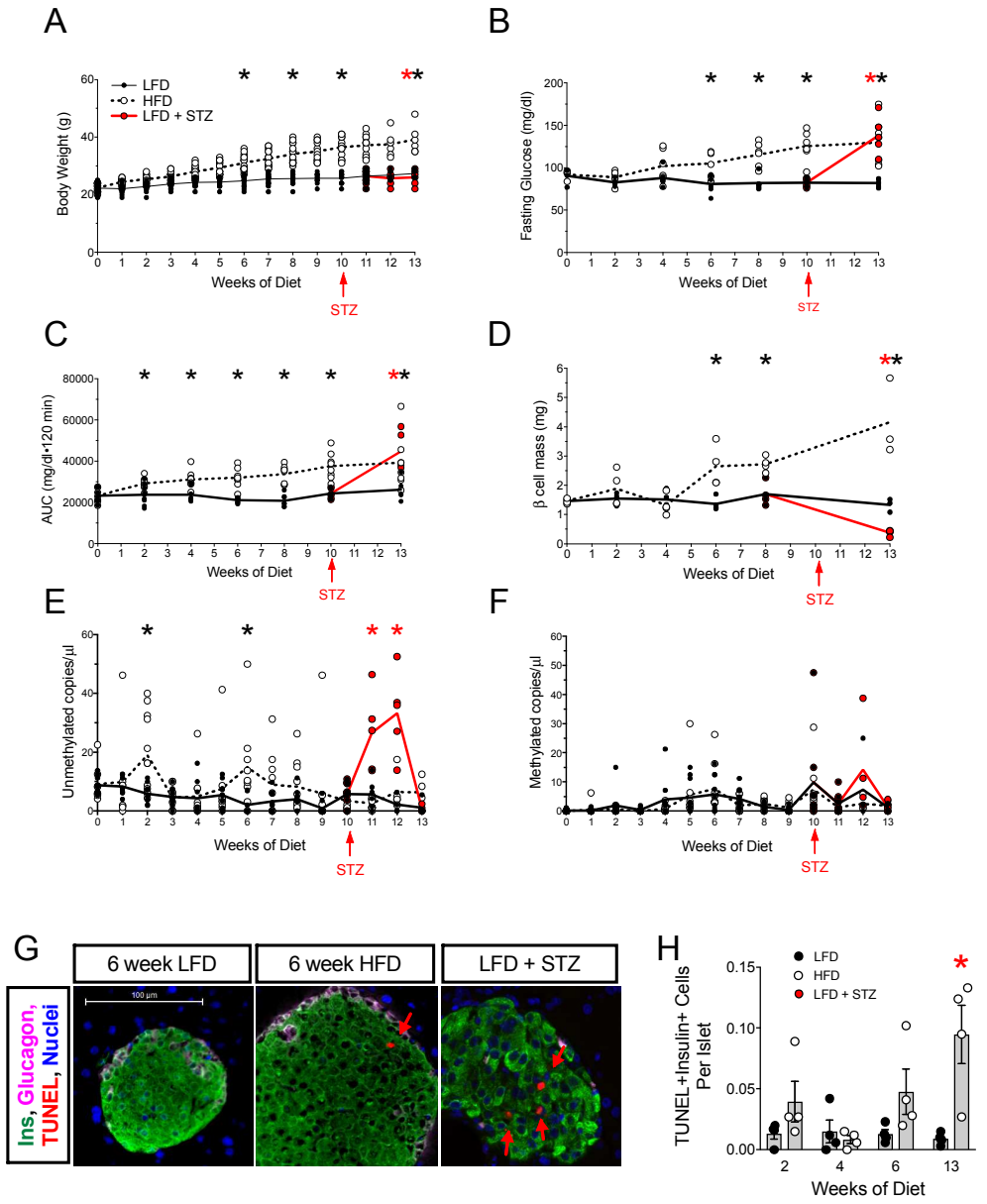


Figure 8: Circulating unmethylated and methylated *Ins2* DNA during LFD and HFD feeding in C57BL/6J mice. Mice were fed a LFD or HFD starting at 8 weeks of age. At 11 weeks on diet, a cohort of mice in each group was treated with STZ to induce β-cell death. (**A**) Body weight measurements; (**B**) Fasting blood glucose measurements; (**C**) Area under the curve of intraperitoneal glucose tolerance tests; (**D**) β-cell mass; (**E**) Circulating unmethylated *Ins2* DNA levels; (**F**) Circulating methylated *Ins2* DNA levels; (**G**) Representative images of pancreata stained for insulin (*green*), glucagon (*pink*), TUNEL (*red*), and DAPI (*blue*); (**H**) TUNEL+/insulin+ cells per islet. N=6-12 mice total for each of 4 groups, done on two separate occasions; N=4 mice for staining, 5 sections per animal. Data are presented as mean ± SEM; **P*<0.05 for HFD compared to LFD or for LFD-STZ compared to LFD.

We serially collected sera from *C57BLKS/J-db/db* (henceforth referred to as "*db/db"*) and control *C57BLKS/J-db/+* (henceforth referred to as "*db/+"*) mice weekly from 6 to 10 weeks of age and subjected them to the DMD assay. As shown in Fig. 9A, *db/db* mice remained significantly hyperglycemic compared to *db/+* controls throughout this timeframe. No statistically significant differences were seen in unmethylated *Ins2* DNA (Figure 9B) nor were differences seen in Insulin+ cells at 5, 7, and 9 weeks (Figure 10), findings that are consistent with prior studies that suggest that β-cell dedifferentiation, rather than loss, may be the major feature in *db/db* mice (181). Methylated *Ins2* DNA exhibited a significant increase in *db/db* compared to *db/+* controls at 7 weeks of age, but returned to control levels thereafter (Figure 7C). Collectively, these mouse studies indicate that β-cell death (as assessed by unmethylated *Ins2* DNA levels) occurs episodically during the development of obesity and dysglycemia in mice and acutely upon β-cell killing by STZ, but persistent β-cell death is not detectable by our DMD assay in a mouse model of established T2D (*db/db*).

III.C. Discussion

Measurement of circulating differentially methylated *INS* DNA has been gaining increasing attention as a minimally invasive biomarker of islet β-cell death that may be used to distinguish individuals with impending and new-onset T1D (186,187,189). However, much less is known about the characteristics of these DNA fragments in the setting of obesity and T2D. In this study, I applied the DMD assay to quantify circulating levels of unmethylated and methylated *Ins2* in mouse models of obesity and T2D. These studies revealed a key finding not previously appreciated, notably that circulating unmethylated *INS*, a biomarker of β-cell death, increases episodically and transiently during the development of obesity and glucose intolerance in mice, but is not persistently elevated in established T2D.

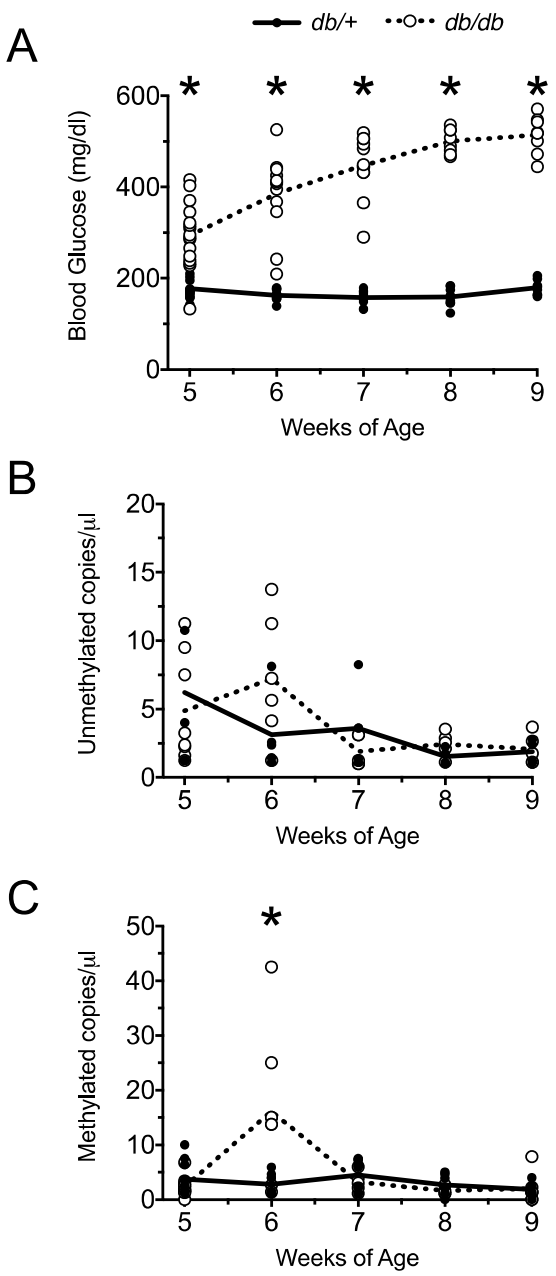


Figure 9: Circulating unmethylated and methylated *Ins2* DNA in *C57BL/KsJ-db/db* mice and controls. Cohorts of *C57BLKS/J-db/db* (*db/db,* N=16) and *C57BLKS/J-db/+* (*db/+,* N=16) were followed between 6-10 weeks of age, and whole blood or serum was collected from 4-16 animals at each time point and assessed for: (**A**) Blood glucose levels, (**B**) Circulating unmethylated *Ins2* DNA levels, (**C**) Circulating methylated *Ins2* DNA levels. Data are shown as mean ± SEM, **P<0.05* compared to corresponding values in *db/+* animals.

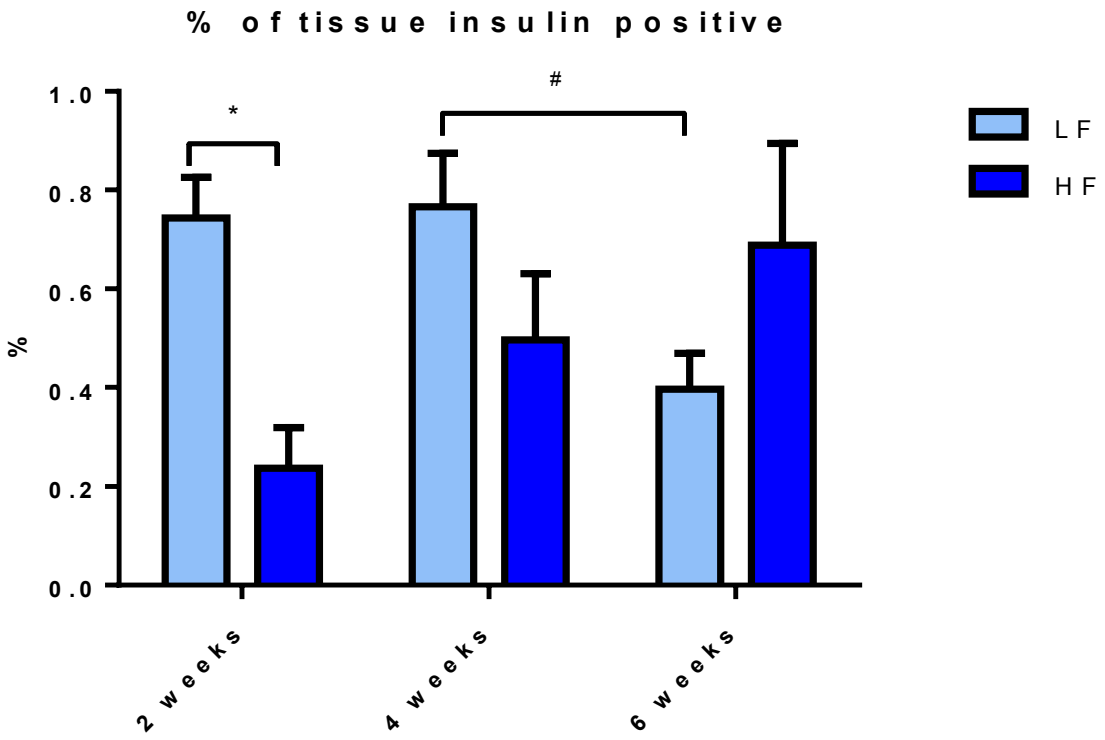


Figure 10: Insulin staining of pancreas. Cohorts of *C57BL6* fed HFD and LFD for 6 weeks. Representative graph of pancreata stained for insulin; N=6-12 mice total for each of 4 groups, done on two separate occasions; N=4 mice for staining, 5 sections per animal. Data are shown as mean ± SEM, **P<0.05* compared to corresponding values of HF, # significantly different compared to other time point, One-Way ANOVA/Tukey's.

The loss of functional β-cell mass is believed to underlie virtually all forms of diabetes, but in T2D the primary etiology remains unclear. Autopsy studies have demonstrated increases in β-cell mass in adults with obesity and pre-diabetes compared to controls, and lower β-cell mass in individuals with frank T2D compared to controls (177,195). The loss in functional β-cell mass in T2D has been attributed variably to β-cell apoptosis and/or dedifferentiation (177,180,195), but definitive evidence for either is lacking. The DMD assay has been utilized by our group and others to monitor active β-cell death in T1D, and provides an opportunity to assess contribution of β-cell death to the pathogenesis of T2D.

Methylation at CpG sites is an epigenetic modification that is correlated with silencing of genes. Therefore, genes that are mostly unmethylated at CpG sites show evidence of being actively or preferentially expressed (196). The mouse *Ins2* gene is predominantly unmethylated at CpG site –182 bp (relative to the transcriptional start site) in islet β-cells, where its expression levels are greatest. In other cell types, *Ins2* is mostly (though not completely) methylated at this site (185,188). Our lab has shown previously that the appearance of elevated unmethylated *Ins2* in the circulation is reflective of dying β-cells that liberate their DNA (184,186). Utilizing ddPCR to measure levels of unmethylated and methylated *Ins2*, I report here that *C57BL/6J* mice placed on a HFD exhibit transient β-cell death (as reflected by increases in unmethylated *Ins2*) corresponding to time points where glucose levels were first increased during GTT (2 weeks of feeding) and at fasting (6 weeks of feeding). The studies in this chapter suggest that measurement of unmethylated *Ins2* may be a more sensitive measure of β-cell death than quantitative tissue staining techniques, since the latter may be limited by sampling error. These findings in HFD-fed mice contrast with those in NOD mice (a model of T1D), where levels of unmethylated *Ins2* DNA appear to remain persistently elevated during the dysglycemic pre-diabetic phase (184). Overtly diabetic *db/db* mice

showed no detectable elevations in unmethylated *INS* DNA levels compared to control *db/+* animals during a 4-week period, suggesting that active β-cell death may not be a major ongoing feature in the setting of established T2D. These findings suggest that β-cell death may be an episodic feature of obesity and dysglycemia. Further evaluation of ER stress gene and protein expression could help elucidate any correlation to these episodic elevations of unmethylated *Ins2* and β-cell ER stress. In the studies presented in this chapter β-cell mass expansion was assessed by determination of β-cell density via immunofluorescence, other cells such as the α-cell within the microenvironment was not measured for expansion as we did not expect changes in this population under the conditions of the experiments. More studies are required to determine correlation between obesity in youth compared to effects in later stages and/or any links with pancreatic growth disorders (i.e. pancreatic cancer).

There are some caveats to the findings in this chapter. First, given the short (~90 min) half-life of DNA in the circulation (187), the episodic nature of β-cell death during obesity progression seen in my mouse models could easily be missed when studying cross-sectional human cohorts with obesity. As such, my data emphasize the important of obtaining *longitudinal* collections in humans to assess the occurrence of β-cell death. Second, my DMD assay differs with respect to the targeted *Ins2* gene CpG site compared to other DMD assays recently described (187–189). Therefore, it is necessary to cautiously compare findings between different assays, even in similar/identical populations (as different CpG sites can exhibit differing degrees of methylation durability with disease progression) (197). Third, and most importantly, my findings only apply to mice, and to date there are no definitive data (one way or the other) to suggest that the reduction in β-cell mass observed in humans with T2D arises from β-cell death. Taken together, my findings in this chapter suggest that unmethylated *Ins2* is a reflection of β-cell death that can be detected in longitudinal cohorts of mice with obesity.

Chapter IV

DHS as a modulator of β-cell replication during compensation

In this chapter, I explore the adaptive response the β-cell undergoes during exposure to high fat diet. In particular, I test how DHS and eIF5A play roles in facilitating cell cycle progression during the compensatory phase of the β-cell to acute and chronic exposure to high fat diet. Experiments with PKCς kinase dead (PKCς-KD) mutant mouse were carried out in collaboration with the lab of Adolfo Garcia-Ocaña at the Icahn School of Medicine at Mount Sinai, New York.

IV.A. Introduction

Although islet β-cells are limited in their ability to replicate, in postnatal life in both humans and rodents they undergo mass expansion to parallel the linear growth of the organism (51,91,198,199). As such, this postnatal period (up to the age of 20 weeks in mice) represents the period of time when β-cell replication and its regulation has been studied. As with any differentiated somatic cell type, most β-cells in the postnatal stage are mitotically quiescent, residing in G0 phase of the cell cycle. Upon mitogenic stimulation (linear growth of the animal, response to insulin resistance, etc.), β-cells enter cell cycle and proceed through the G1 phase, preparing for DNA replication. The transition from the G1 to S phase, in which cells double their DNA content, is the first "checkpoint" of the cell cycle, and as such is a regulated process (200). Subsequent passage through G2, then the M phase (the mitotic phase) allows the cellular components previously produced to be divided between the two daughter cells (201). The G2-M transition is also regulated in many cell types, and is considered a second "checkpoint" (202). Key proteins that regulate these checkpoints are the cyclin-dependent kinases (CDKs). These proteins associate with other molecules, known as

cyclins, throughout the cell cycle to ensure timely progression (203). CDKs in turn are tightly regulated by CDK inhibitors to further safeguard the cell from progression of cell cycle in unfavorable circumstances (204).

In both rodents and humans, cyclin-D proteins are crucial in the progression of islet β-cells through the cell cycle. In rodents, an especially important role of cyclin D2 was first described in the context of the normal postnatal β-cell expansion during linear growth of the animal. Mice deficient for cyclin D2 have impaired β-cell expansion and function (205). Cyclin-D1-deficient mice are small, with impaired neuronal, retinal, and mammary gland development (206,207), but appear to have no gross defects in β-cell replication per se (205). Cyclin-D3-deficient mice have reduced adipocyte size and increased sensitivity to insulin, but no obvious β-cell phenotype (208). Cyclin-D activity accumulates in G1, as a function of mitogenic signaling. Restraining the conversion of glucose to lipids for storage by the cyclin-Ds allows the cell to use glucose-produced metabolites for production of cell content for cell division to take place (209). Alternatively, metabolism inhibition by the cyclin-Ds might provide a negative feedback to ensure unidirectional cell cycle progression. If metabolic activity increases cyclin-D expression, and cyclin-Ds inhibit metabolism, a rise in cyclin-D in G1 would shut down metabolism, therefore preventing re-entry into G1 until G1/S transition has completed.

As noted above, cyclin-D2 is essential for normal postnatal β-cell growth and consequent glucose homeostasis in the growing animal. *CyclinD2*$^{-/-}$ mice develop diabetes owing to inadequate β-cell replication and mass (205). In addition, cyclin-D1 appears to play a supportive role, since complex knockout/*heterozygous cyclinD2*$^{-/-}$ */cyclinD1*$^{+/-}$ mice show a much more severe β-cell growth deficiency than animals lacking either factor alone, resulting in death due to severe ketoacidosis as early as 4 months of age (205). Strikingly, however, these cyclins are not essential for β-cell proliferation

during pancreas development, since β-cell mass at birth appears normal in their absence (205).

Feeding rodents a high fat diet (HFD) increases β-cell mass (69). The timing and identity of the enabling factors of the murine compensatory growth response are not comprehensively known, but it does appear that cyclin-D2 is essential in this process (210–212). Longitudinal studies in mice show that β-cell mass expands as insulin demand increases, and eventually declines as diabetes develops (213). The increase of β-cell mass could occur as a result of cell replication, hypertrophy, and/or neogenesis (214). Replication is believed to be the dominant mechanism for adult β-cell mass growth in mice (215), and by comprehensively cataloging these signals, the limitations to replication in adult β-cells could be ascertained.

In the context of pathways that promote cell cycle entry, the growth factor signaling/mammalian target of rapamycin (mTOR) pathway has seen perhaps the greatest attention (210,211,216–219). However, a pathway that has received little attention has been the polyamines/eukaryotic translation initiation factor 5A (eIF5A) pathway. Polyamines (putrescine, spermidine, spermine) and eIF5A have been extensively studied in the context of oncogenesis, as these factors promote cellular replication by enabling the translation of intracellular mRNAs during cellular growth (220,221). Spermidine links the polyamines to eIF5A via the enzyme deoxyhypusine synthase (DHS). DHS is the rate-limiting enzyme that transfers an aminobutyl moiety from spermidine to the ε-amino group of Lys50 of eIF5A, to form the amino acid hypusine (hydroxyputrescine lysine) (221). This modification is required for all the known mRNA translational functions of eIF5A (222–224).

The precise function of eIF5A in mRNA translation in mammals is still not well understood, but may be pleiotropic. Roles for eIF5A in ribosomal protein synthesis, RNA transport, mRNA stability, and polypeptide chain elongation have all been reported

(144,167,225–230). In the context of the β-cell, eIF5A appears to be important in the production of pro-inflammatory molecules and production of stress-responsive proteins in response to cytokine signaling and activation of the endoplasmic reticulum (ER) stress cascade (144,167,231). However, no specific role for the polyamines or eIF5A have been ascribed to β-cell replication. In this chapter, I describe studies interrogating the role of the hypusine modification in compensatory β-cell replication. Using a novel, β-cell-deficient mouse model of DHS, I show that DHS is necessary for the proliferative response of β-cells to HFD feeding, and that the effect can be attributed to a specific role for DHS/eIF5A in enabling cyclin-D2 mRNA translation.

IV.B. Results

Generation of β-cell-specific Dhps knockout mice (*Dhps*Δ*β*)

As shown in Figure 11A, our lab floxed exons 2-7 of the gene encoding DHS (*Dhps*) in mice. Identification of mice harboring the floxed *Dhps* allele was identified by genotyping, as shown in Figure 11B. I generated mice in which the *Dhps* gene was specifically and inducibly deleted in β-cells, by crossing *Dhps*$^{loxP/+}$ mice to *MIP1-CreERTM* mice (232) and breeding to homozygosity for deletion. To induce deletion of the *Dhps* gene postnatally, tamoxifen was administered to cohorts of animals at postnatal age of 8 weeks. I selected 8 weeks of age as a timepoint to induce DHS knockout since animals show evidence of β-cell replication at this age (199), therefore changes in mass and/or function of β-cells would be more noticeable. *Dhps*Δ*β* mice and control littermates were allowed to acclimate for 1 week prior to further experimentation. To determine knockout specificity for β-cells, mice were euthanized two weeks after tamoxifen administration and islets, brain, liver and muscle were isolated. As expected, DHS protein levels were significantly reduced in the islets of the *Dhps*Δ*β* mice compared to controls, and was unaffected in the liver and brain tissues tested (Figure 11C, D and

E). To further confirm the specificity of the recombination, a floxed Tomato fluorescent reporter gene in the *Rosa26R* locus was crossed into the breeding line. As shown in Figure 11F, recombination was observed only in β-cells by immunofluorescence staining of insulin by rabbit antibody and nuclear fluorescence of tomato+ cells (though the efficiency appears to be 50-60% of β-cells, a finding consistent with the known efficiency of the Cre driver strain via quantification of tomato+insulin+ of total insulin+ cells). To assess the metabolic impact of deletion of *Dhps* in β-cells, I measured body weight and glucose tolerance 1 week after tamoxifen treatment. As depicted in Figure 11G, no differences in weight between the *Dhps*Δ*β* mice and the control littermates were observed. Additionally, *Dhps*Δ*β* mice exhibited no differences in glucose tolerance when compared to the control littermates (Figure 11H). These studies suggest that deletion of *Dhps* in the β-cell does not acutely affect metabolic control.

*Loss of glucose tolerance and β-cell mass in Dhps*Δ*β mice.*

To test the role DHS in β-cell mass expansion, I next followed *Dhps*Δ*β* mice and their littermates on both a normal chow diet (NCD, 16% kcal from fat) and on a high fat diet (HFD, 60% kcal from fat), as shown in the schematic in Figure 12A. NCD-fed *Dhps*Δ*β* mice appeared to exhibit a slight worsening of glucose tolerance after 4 weeks in the diet compared to the littermate controls (Figure 12B), however, this did not approach statistical significance by AUC analysis (Figure 12D). These data suggest that the loss of *Dhps* in β-cells has only a minimal impact on the normal growth responses of β-cells in young animals. By striking contrast, knockout of *Dhps* led to evidence of frank diabetes compared to HFD-fed littermate controls based on fasting glucose measurements and glucose measurements following an intraperitoneal glucose load (Figure 12C and D). To further evaluate the cause of the glucose intolerance seen in the HFD-fed *Dhps*Δ*β* mice, I conducted *in vivo* glucose stimulated insulin secretion assays

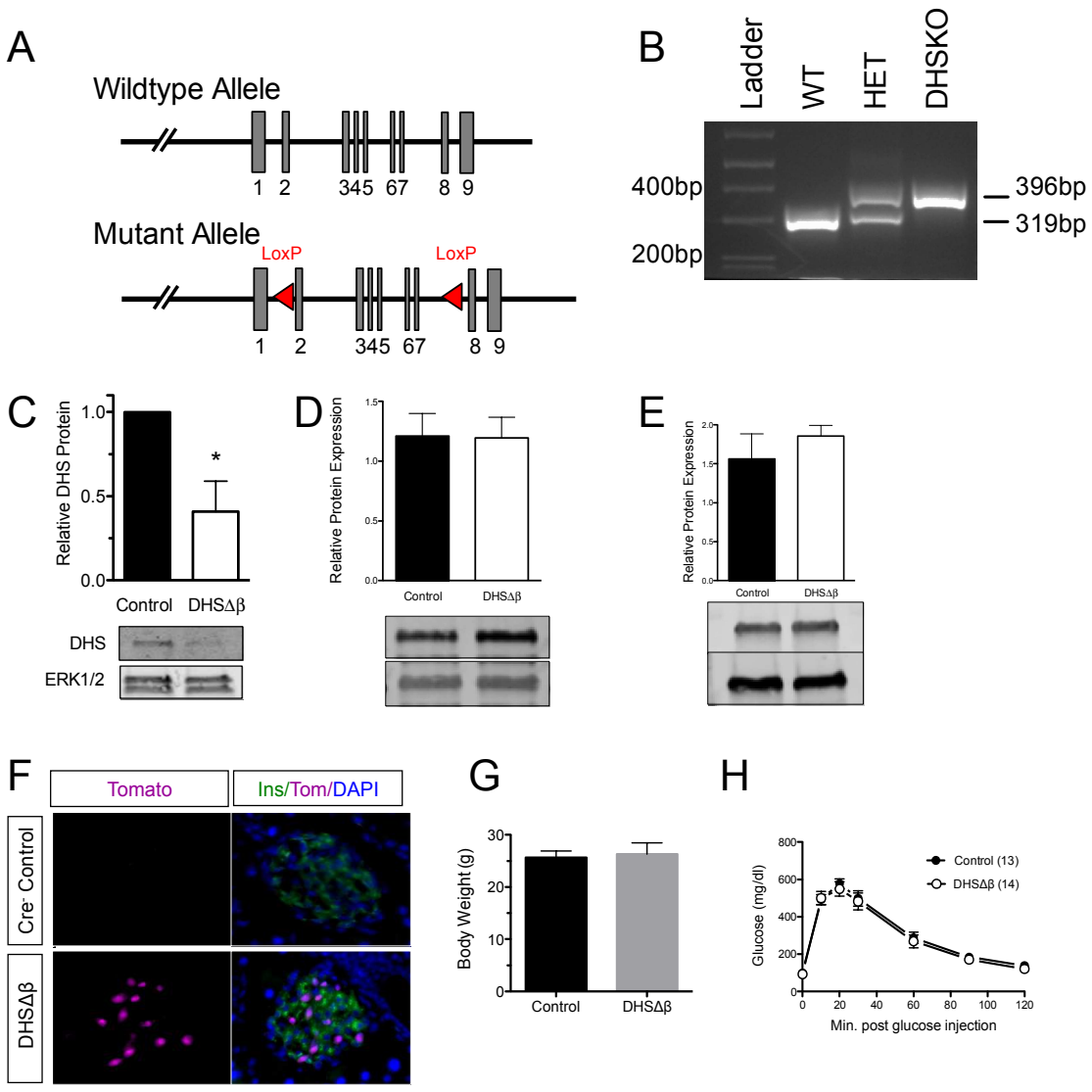


Figure 11: DHS Knockout mouse model is normoglycemic under basal conditions. DHS $^{flox/flox}$ mice were crossed with MIPCre^{RTm} to obtain pancreatic islet beta cell specific DHS knockouts after 5mg Tamoxifen administration at 8 weeks of age. The model shows specificity to the islet. Mice showed no physiological effects following the knockout. (**A**) Schematic diagram of the *Dhps* gene targeting in flox/flox and wild-type mouse locus; (**B**) Electrophoresis gel of genotyping confirming DHS knockout; (**C**) Immunoblot of DHS protein levels in the pancreatic islet 2 weeks after Tamoxifen; (**D**) Immunoblot of DHS protein levels in the brain 2 weeks after Tamoxifen; (**E**) Immunoblot of DHS protein levels in the liver 2 weeks after Tamoxifen; (**F**) Immunofluorescence of DHS knockout mice carrying *Rosa-26* Promoter expression; (**G**) Body weight measurement after 2 weeks of Tamoxifen administration; (**H**) Glucose Tolerance Test after 2 weeks of Tamoxifen injection. N=3 to 14 mice total. Data are presented as mean ± SEM; **P*<0.05 for DHSΔβ compared to control.

(GSIS). Fig. 12E shows that insulin levels in *Dhps*Δ*β* mice are similar to HFD-fed littermate controls after an overnight fast, but show significantly impaired insulin levels in response to the intraperitoneal glucose load.

To determine if the glucose intolerance and reduced insulin response was due to decreased number of β-cells, I next examined β-cell mass in HFD-fed *Dhps*Δ*β* mice and control littermates. Compared to control HFD-fed mice, HFD-fed *Dhps*Δ*β* mice had significantly reduced (about 2-fold) β-cell mass (Figure 12F). The reduction in β-cell mass in HFD-fed *Dhps*Δ*β* mice could have resulted from either increased β-cell death or reduced β-cell proliferation. To determine if the lack of β-cell compensation was due to increased β-cell death, I used the DMD assay (as presented in Chapter III) to measure β-cell death in HFD-fed control and *Dhps*Δ*β* mice. As shown in Figure 13A-D, I did not see an increase in β-cell death *Dhps*Δ*β* mice compared to control animals at either the 1-week or 4-week timepoints on the HFD, raising the likelihood that DHS impacts proliferation of β-cells.

In pursue of analyzing pathways that are affected by DHS deficiency in the β-cells, high-throughput sequencing technology (RNA-seq) was utilized. RNA of isolated islets from mice fed HFD for 4 weeks was submitted to the Center for Medical Genomics in Indiana University School of Medicine. As expected, I found RNAs involved in cell growth pathways to be affected in the *Dhps*Δ*β* mice compared to controls (Figure 14). The gene expression affected in HF-fed *Dhps*Δ*β* mice compared to controls included downregulation of genes involved in DNA replication, mitotic nuclear division and regulation of developmental growth. Some of these genes found are: Neurofibromin 2 (Nf2), Transforming growth factor beta 1 (Tgfb1), minichromosome maintenance complex component family (MCM2), checkpoint kinase 2 (Chek2), cell division cycle 45 (Cdc45), cyclin-dependence kinase 1 (Cdk1), cyclin-dependent kinase inhibitor 1A (Cdkn1a), and Igf1 to mention a few. The misexpression of these genes could be the

result of β-cell compensation being impacted by the deficiency of DHS under HFD exposure.

Increased glucagon expression after 4 weeks of HFD feeding in Dhps∆β mice

In addition to the limitation on processes of β-cell survival, β-cell dedifferentiation has been observed *in vitro* and *ex vivo* experiments (233,234). There is evidence of its occurrence in partial pancreatectomy studies, however, the possibility of occurring in T2D is yet to be explored. To evaluate if there are any changes on cell morphology in *Dhps∆β* mice after different time-points of HFD feeding. Pancreata of *Dhps∆β* and control mice was evaluated with confocal immunofluorescence using immunohistochemistry for insulin and glucagon hormone expression. I observed no differences in cell morphology and hormonal expression in the pancreata of 1 week HF-fed mice (Figure 15A). However, glucagon expression was observed throughout the islet of 4 week HF-fed *Dhps∆β* mice whereas the controls had no detectable differences in glucagon expression (Figure 15B). The appearance of more glucagon expression in the 4 week HF-fed *Dhps∆β* mice provide an explanation as to why these mice cannot compensate at this stage even though they were able to keep euglycemia at 1 week of HFD.

Defects in β-cell proliferation are seen early following HFD feeding in Dhps∆β mice

Compensatory gains in β-cell mass in response to HFDs are reflected as increases in β-cell proliferation as early as 1 week following initiation of HFDs (54,211,212,235). To clarify the role of DHS in β-cell compensation, we therefore examined glucose tolerance, β-cell proliferation and β-cell mass in control and *Dhps∆β* mice after 1 week of NCD or HFD feeding. Notably, HFD-fed *Dhps∆β* mice exhibited significantly improved glucose tolerance compared to controls following 1 week of

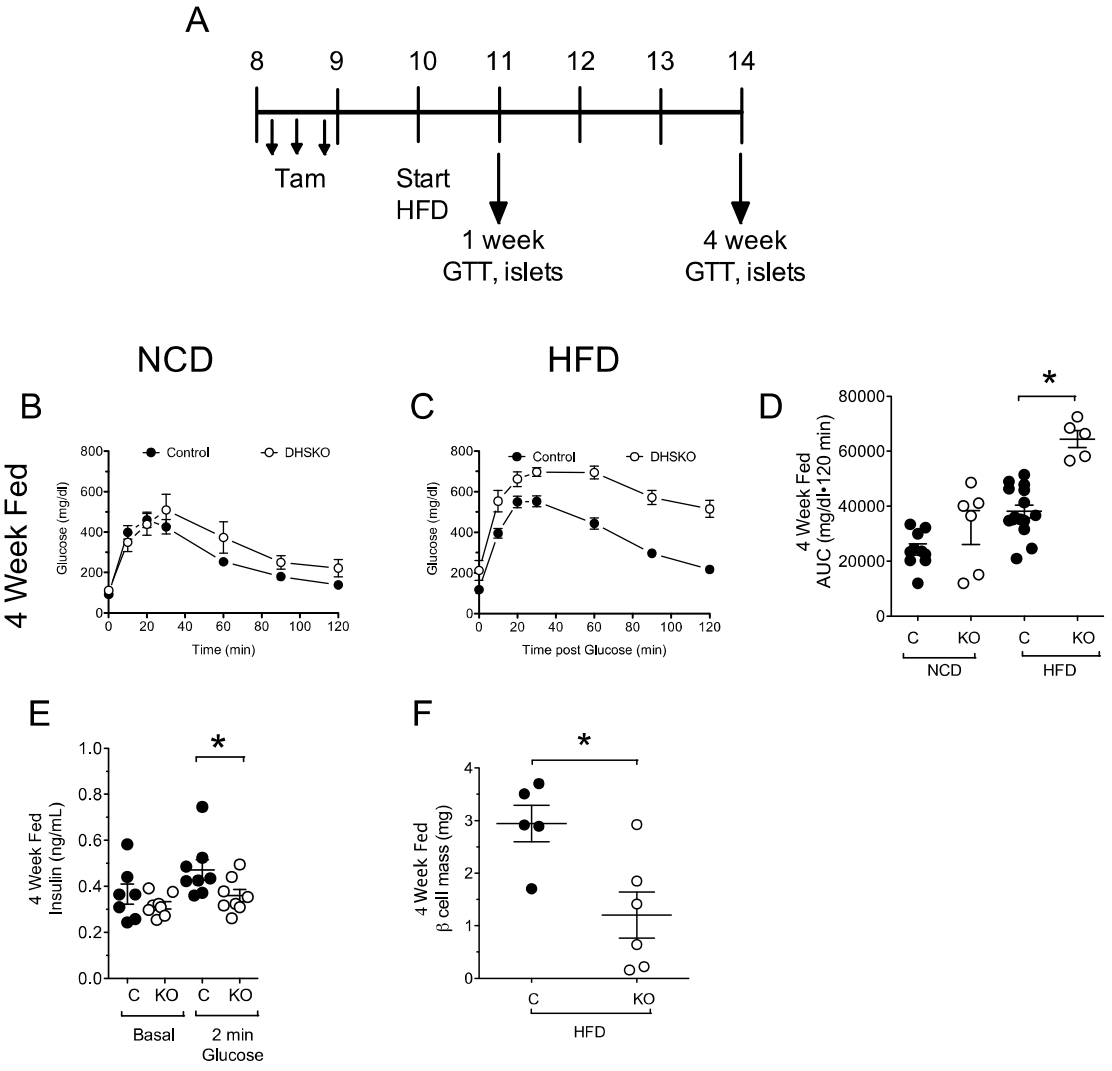


Figure 12: Loss of DHS in the islet beta cell results in impaired glucose tolerance and beta cell mass decline. DHSΔβ and control mice were subjected to 4 weeks of HFD feeding from 10 weeks of age. Mice showed impairment of glucose clearance via GTT, reduction of insulin secretion and decline beta cell mass. (**A**) Schematic diagram of feeding schedule of DHSΔβ and control mice after Tamoxifen administration; (**B**) GTT of mice fed a normal chow diet (NCD); (**C**) GTT of mice fed a high fat diet (HFD); (**D**) Area under the Curve (AUC) of GTT; (**E**) Insulin ELISA of serum *in vivo* GSIS; (**F**) Beta cell mass calculation after 4 weeks of diet. N=3 to 14 mice total. Data are presented as mean ± SEM; **P*<0.05 for DHSΔβ compared to control.

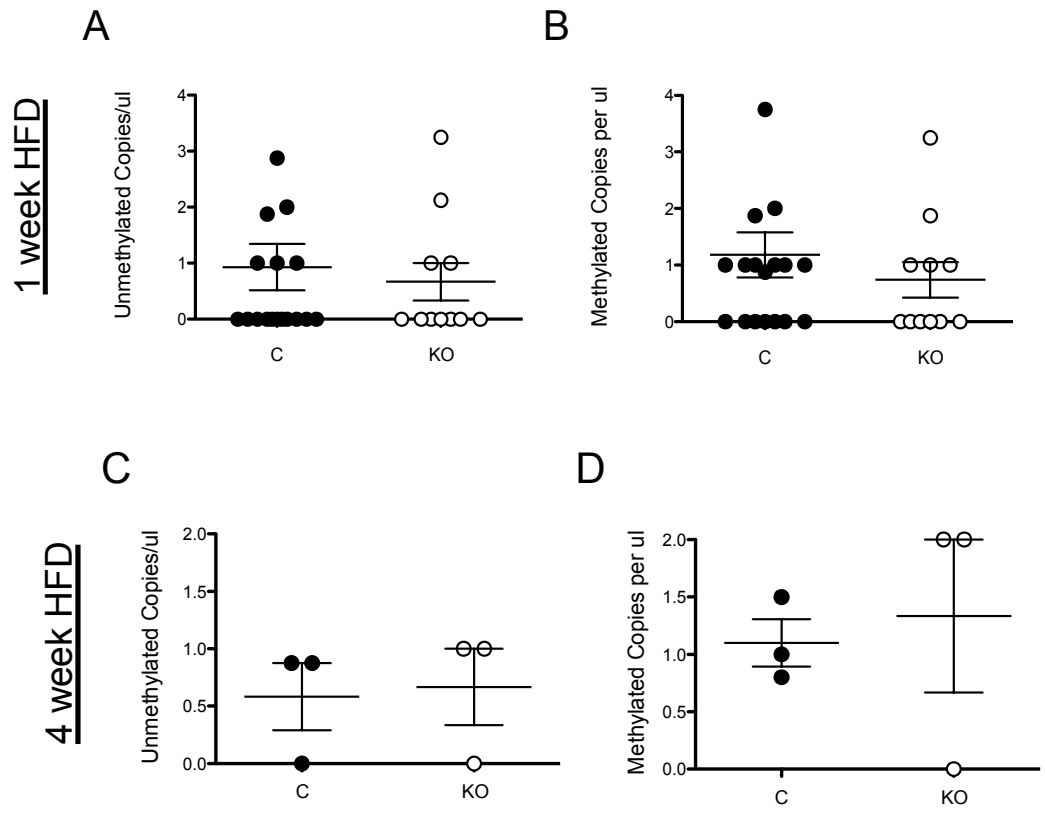


Figure 13: Circulating unmethylated and methylated *Ins2* DNA during HFD feeding in DHSΔβ mice. Mice were fed a HFD starting at 10 weeks of age. A cohort of mice was sacrificed after 1 week on HF and another small cohort after 4 weeks of HFD. (**A**) Unmethylated copies of circulating *Ins2 DNA* in the serum after 1 week of HFD; (**B**) Methylated copies of circulating *Ins2 DNA* in the serum after 1 week of HFD; (**C**) Unmethylated copies of circulating *Ins2 DNA* in the serum after 4 weeks of HFD; (**D**) Methylated copies of circulating *Ins2 DNA* in the serum after 4 weeks of HFD. N=3-12 mice total for each group.

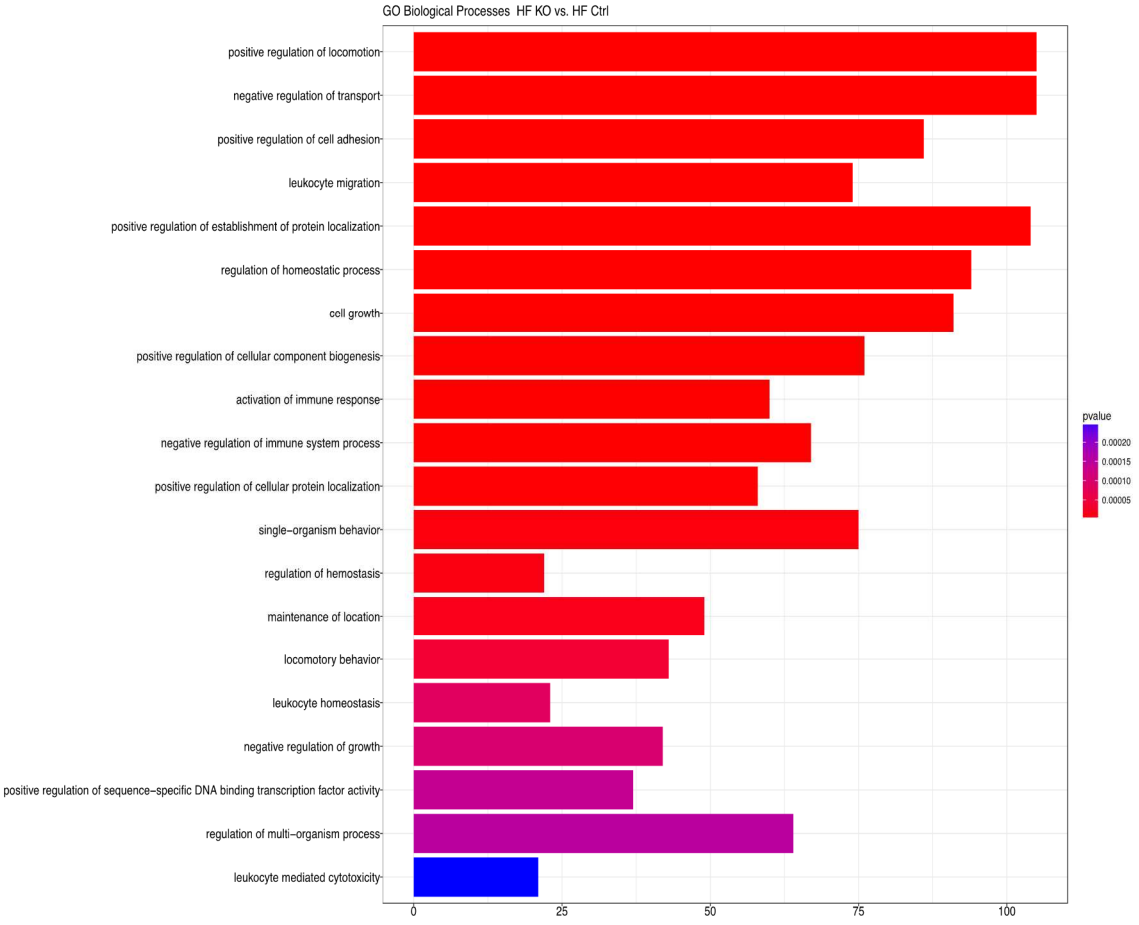


Figure 14: Cell growth impaired in DHSΔβ mice. RNA-seq of isolated islets from 4 week HFD-fed DHSΔβ mice. N=3-5. *P<0.05 compared to CTL.

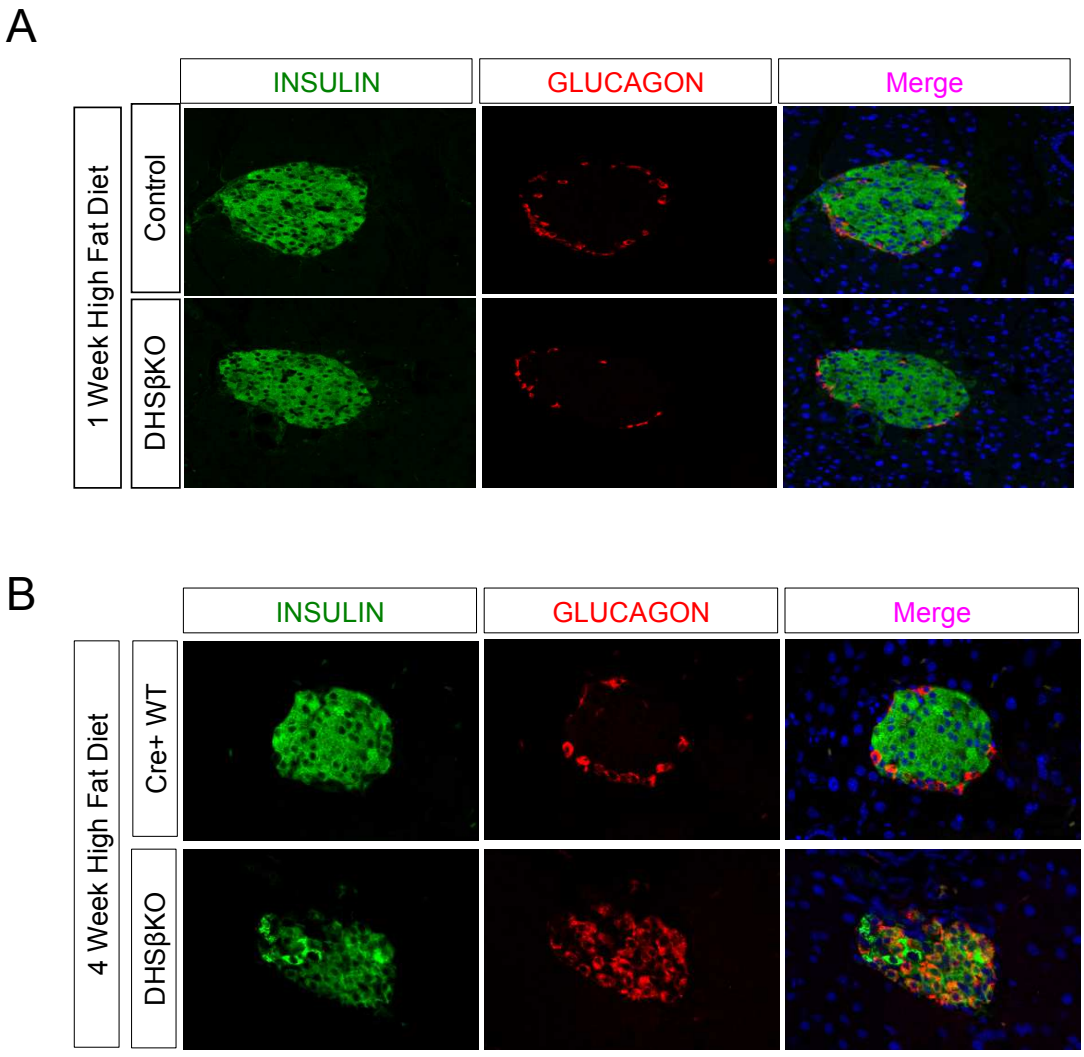


Figure 15: Cell morphology changes after 4 weeks of HFD in DHSΔβ mice. Mice were fed a HFD starting at 10 weeks of age. A cohort of mice was sacrificed after 1 week on HF and another small cohort after 4 weeks of HFD. (**A**) Immunofluorescence of insulin and glucagon in DHS knockout mice after 1 week of HFD; (**B**) Immunofluorescence of insulin and glucagon in DHS knockout mice after 4 week of HFD.

feeding (Figure 16A, B and C). Consistent with this finding, circulating insulin levels in HFD-fed *Dhps∆β* mice were significantly higher than the controls (Figure 16D). At 1 week of HFD, β-cell mass was not different between *Dhps∆β* and control mice (Figure 16E). On a NCD, no differences in glucose tolerance, circulating insulin, and β-cell mass were observed between *Dhps∆β* mice and littermate controls (Figure 16A-E).

To assess the role of DHS in β-cell proliferation during the compensatory stage of the β-cells, I first examined the activity of DHS by performing immunofluorescence using a custom hypusine-specific antibody created by our lab (236). $EIF5A^{Hyp}$ levels were increased in islets after HFD-feeding in control mice, and appropriately reduced/absent in the *Dhps∆β* mice (Figure 17A). Next, we evaluated histological sections for proliferative cell nuclear antigen (PCNA). Whereas 1 week of HFD feeding increased the frequency of insulin+/PCNA+ cells compared to NCD in control littermates as expected, this increase was not apparent in *Dhps∆β* mice (Figure 17B and C). Taken together, these results suggest that DHS deficiency allows increased responsiveness of β-cell insulin secretion but is required for the early β-cell proliferative response to a HFD.

Impaired cyclin-D2 mRNA translation in Dhps∆β HFD-fed mice.

To understand better the potential defects in cell cycle proteins that contribute to the phenotype of Dhps∆β mice, we evaluated mRNA levels for molecules involved in cell cycle progression at the G1/S boundary. We observed no differences in levels of several cell cyle mRNAs, including *Ccnd2* (encoding cyclin-D2), *Ccnd1* (encoding cyclin-D1), and *Ccna2* (encoding cyclin-A2) in Figure 18A. Given the importance of cyclin-D2 in the early proliferative response to HFDs, we were surprised to see no differences in its mRNA in Dhps∆β mice. We therefore next evaluated cyclin-D2 protein levels in islets, and as shown in Figure 18B and C, cyclin-D2 protein levels were significantly reduced in

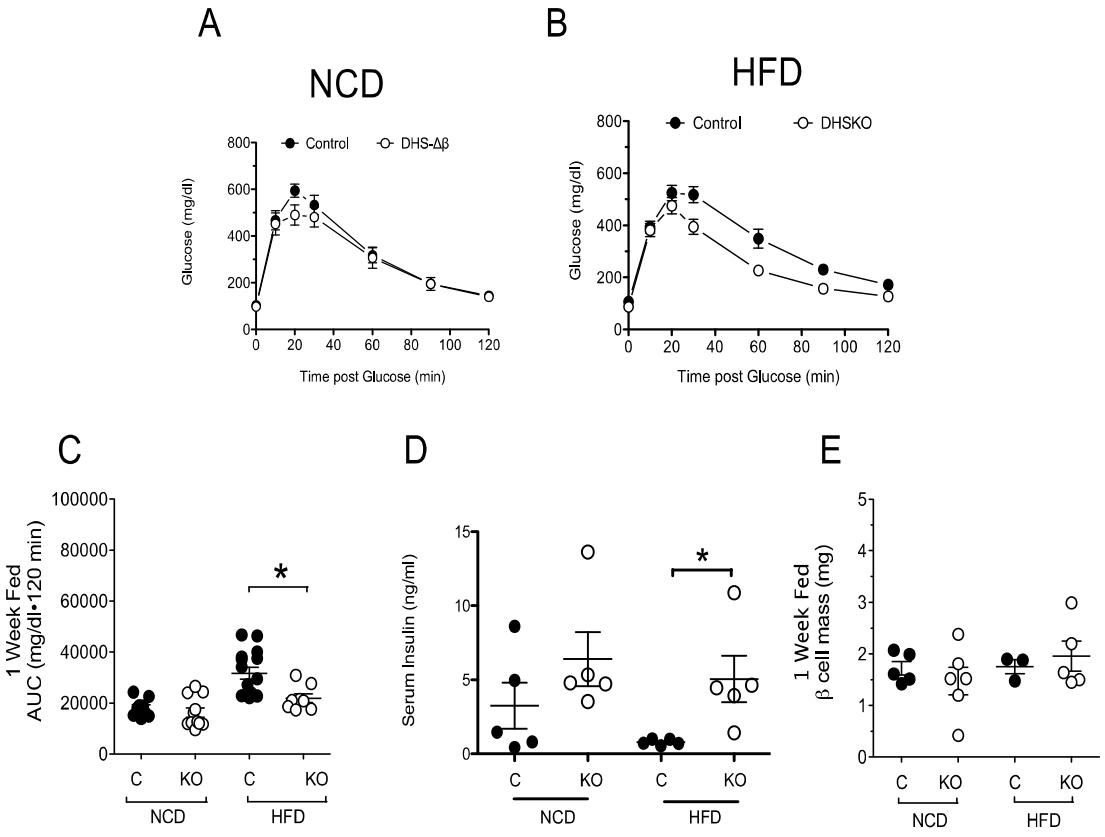


Figure 16: Improved insulin secretion in DHS deficient β-cells mice after 1 week of HFD. DHSΔβ and control mice were subjected to 1 week of HFD feeding from 10 weeks of age. DHSΔβ mice showed no glucose clearance impairment via GTT, and had more serum insulin compared to controls. (**A**) GTT of mice fed a normal chow diet (NCD); (**B**) GTT of mice fed a high fat diet (HFD); (**C**) Area under the Curve (AUC) of GTT; (**D**) Insulin ELISA of random serum insulin; (**E**) Beta cell mass calculation after 1 weeks of diet. N=3 to 14 mice total. Data are presented as mean ± SEM; **P*<0.05 for DHSΔβ compared to control.

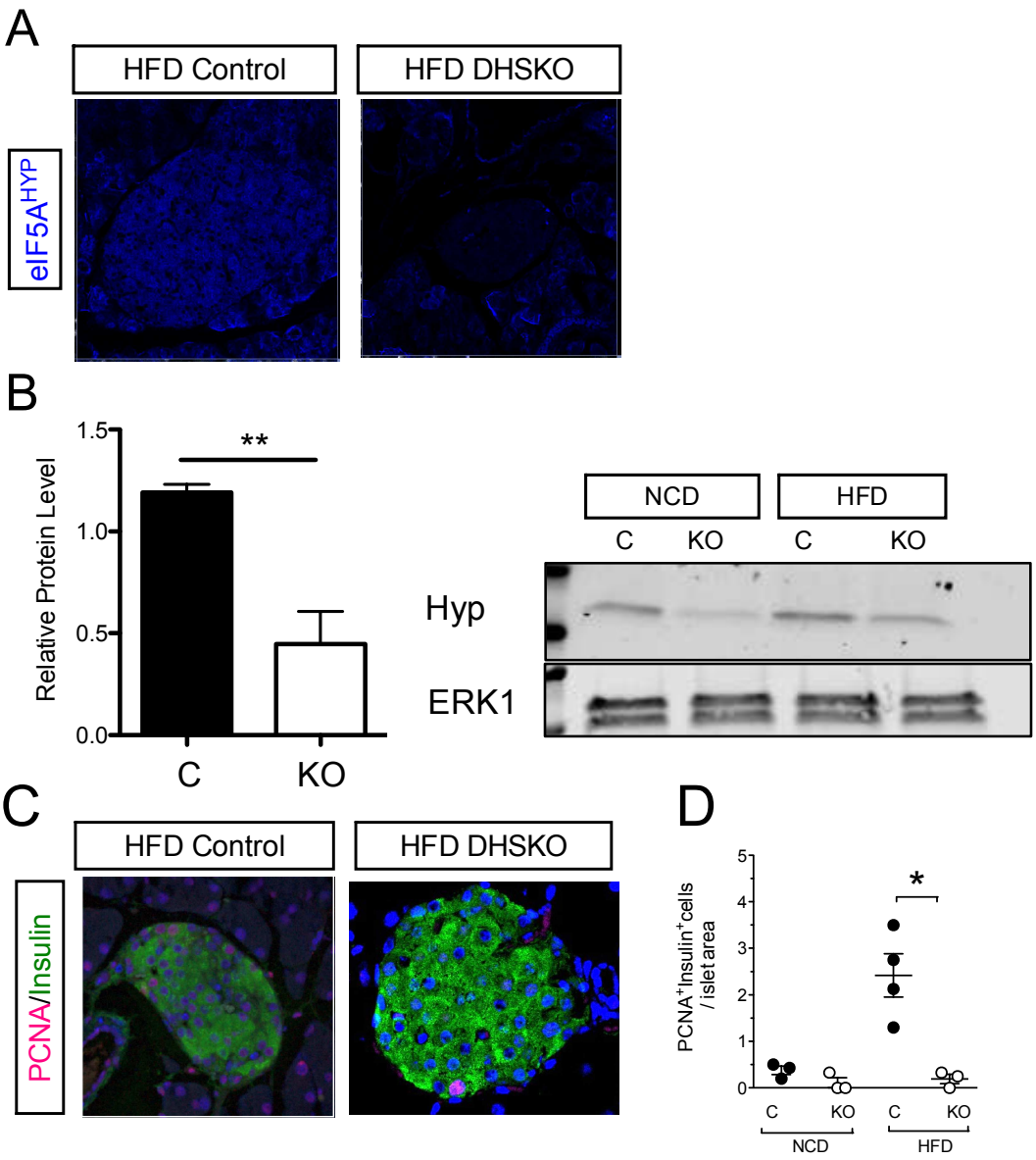


Figure 17: DHS deficiency results in an impaired β-cell proliferative response following 1 week HFD. DHSΔβ and control mice were subjected to 1 weeks of HFD feeding from 10 weeks of age. Mice showed no changes in overall beta cell mass, however, no induction of proliferation. (**A**) Immunofluorescence of eIF5A^{Hyp} of 11 week old mice fed 1 week of HFD; (**B**) Western Blot of eIF5A^{Hyp} of 11 week old mice fed 1 week of NC and HF diets. Protein quantification of HF fed isolated islets; (**C**) Immunofluorescence of PCNA; (**D**) Quantification of PCNA and insulin positive cells. N=3 to 4 mice total. Data are presented as mean ± SEM; **P*<0.05; ***P*<0.001 for DHSΔβ compared to control.

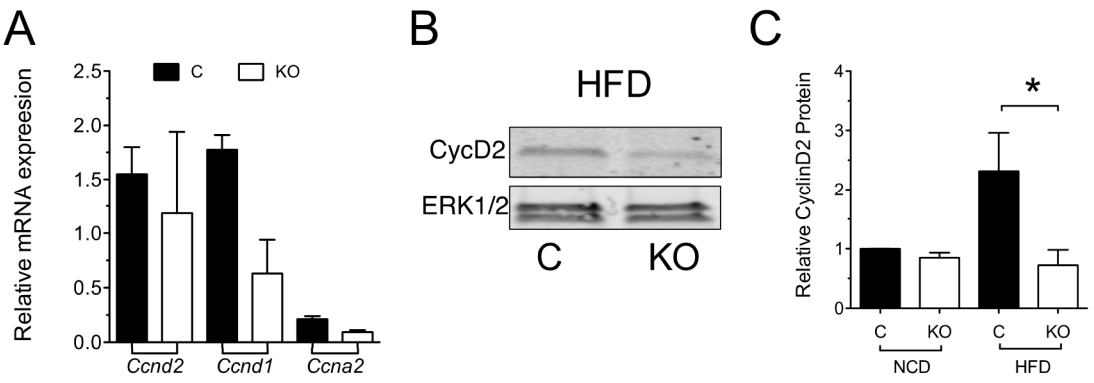


Figure 18: Cyclin-D2 protein synthesis downregulated in *Dhps*Δ*β* mice. *Dhps*Δ*β* and control mice were subjected to 1 weeks of HFD feeding from 10 weeks of age. (**A**) qPCR isolated islets of mRNA; (**B**) Western Blot of Cyclin-D2 for 1 week HFD fed mice; (**C**) Protein levels of isolated islets of 1 week HFD fed mice for mRNA of Cyclin-D2. N=3 to 5 mice total. Data are presented as mean ± SEM; **P*<0.05 for DHSΔβ compared to control.

*Dhps*Δ*β* islets following HFD feeding. Collectively, these data suggest that DHS is necessary for the post-transcriptional regulation of cyclin-D2 levels in the β-cell.

To determine if posttranscriptional regulation of cyclin-D2 levels is at the level of mRNA translation (and a possible role for eIF5A^{Hyp} at this level) we performed polyribosome profile (PRP) experiments on isolated islets of 1-week HFD-fed animals. PRP analysis involves sucrose gradient sedimentation of total RNA from islets, followed by monitoring of RNA distribution across the gradient by UV absorbance. RNAs associated with different components of the ribosome assembly complex sediment differentially, such that those associated with monoriboosomes sediment early, and those that are associated with multiple ribosomes (polyribosomes)—and hence are actively translated—sediment later in the gradient (237,238). We found the overall profile of *Dhps*Δ*β* islets to be unchanged compared to control islets, with the "polyribosome-monoribosome (P/M) ratio" (reflecting the relative association of RNAs with polyribosomes to monoribosomes) to be statistically unchanged (Figure 19A and B). This result suggests that the global translational profile of the RNAs are unaffected by deletion of *Dhps* in β-cells, a finding consistent with the absence of a role for DHS/eIF5A in generalized mRNA translation. However, to interrogate the translation of specific mRNAs, I next collected fractions from the sedimentation gradient and isolated total RNA, and subjected them to RT-PCR for cyclin-Ds. As shown in Figure 19C and D, the mRNA encoding cyclin-D2 (*Ccnd2*) exhibits a clear shift in occupancy toward monoribosomes in *Dhps*Δ*β* islets compared to control islets, indicating an absence of engagement of this mRNA with polyribosomes in *Dhps*Δ*β* islets. By contrast, no changes in the engagement of *Ccnd1* mRNA was observed in DhpsΔβ islets as shown in Figure 19E and F. Collectively, these results suggest that the impaired proliferative response in islets of HFD-fed *Dhps*Δ*β* mice is due to impaired translational engagement *of Ccnd2*.

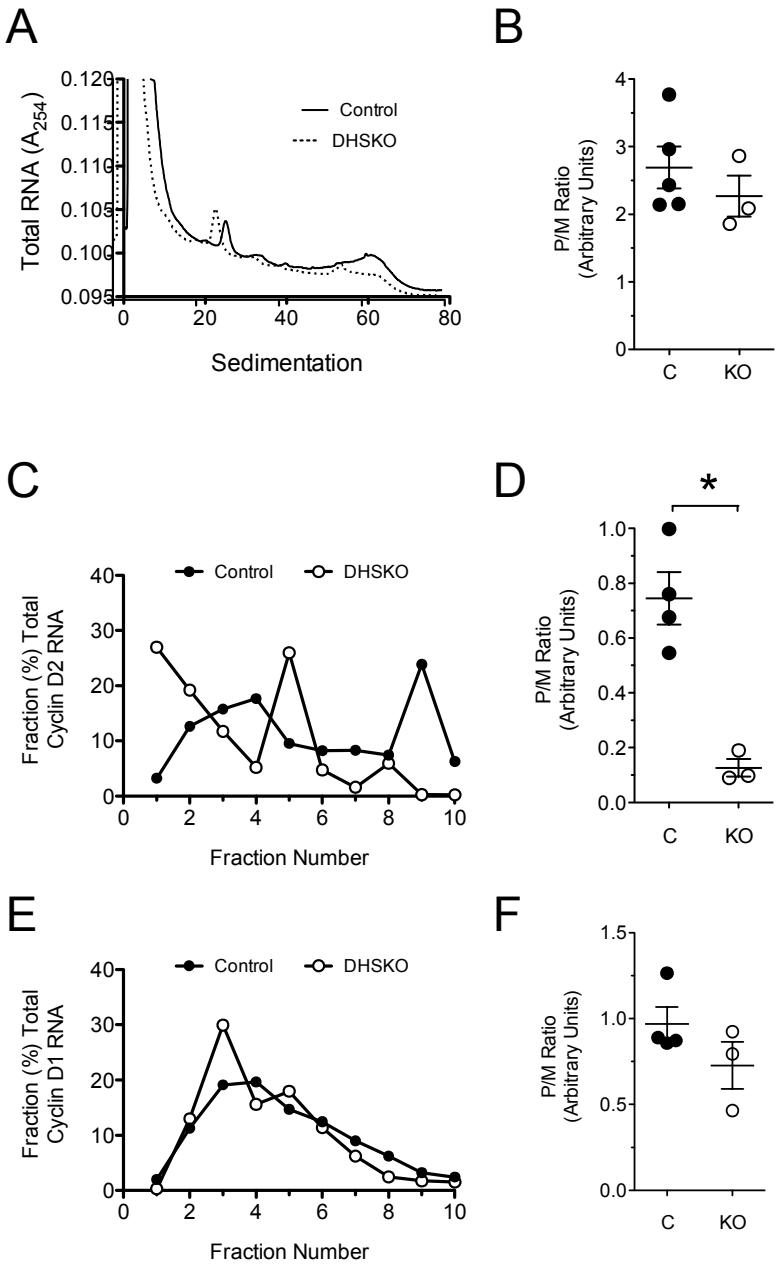


Figure 19: Cyclin-D2 protein synthesis halted by translation initiation block in *Dhps*Δ*β* mice. DHSΔβ and control mice were subjected to 1 weeks of HFD feeding from 10 weeks of age. (**A**) PRP of isolated islets from DHSKO and Ctl treated 1 week HFD; (**B**) P/M Ratio of transcriptome of isolated islets; (**C**) qRT PCR of fractions from PRP for mRNA of Cyclin-D2 ; (**D**) P/M Ratio of fractions for Cyclin-D2; (**E**) qRT PCR of fractions from PRP for mRNA of Cyclin-D1; (**F**) P/M Ratio of fractions for Cyclin-D1. N=3 to 5 mice total. Data are presented as mean ± SEM; **P*<0.05 for DHSΔβ compared to control.

The DHS/eIF5A pathway lies downstream of PKCζ activation, and in parallel to mTOR regulation of mRNA translation.

As noted previously, a key pathway that links extracellular mitogenic signals to activation of mRNA translation and β-cell proliferation is the growth factor/mTOR pathway (210,211,216–219). To determine how the growth factor/mTOR pathway might be linked to polyamines/DHS/eIF5A pathway, we collaborated with the lab of Dr. Adolfo Garcia-Ocaña at the Icahn School of Medicine at Mount Sinai. The kinase PKCζ lies proximal to mTORC1 (239,240), and in mice harboring kinase-dead-PKCζ (KD-PKCζ) protein in β-cells (β-KD-PKCζ), the proliferative response to obesity and insulin resistance is impaired, similar to *Dhpsβ* mice (211). First, we examined the levels of $eIF5A^{Hyp}$ in the islets of HFD-fed β-KD-PKCζ mice by immunoblot. As shown in Figure 20A, $eIF5A^{Hyp}$ levels were increased in control animals fed a HFD, consistent with our own data (Figure 17B). However, $eIF5A^{Hyp}$ levels did not increase in islets upon HFD feeding of β-KD-PKCζ mice (Figure 20A). These findings suggest that PKC ζ loss may impact DHS/eIF5A, and suggests that the phenotype of β-KD-PKCζ mice maybe referable to the loss of hypusine formation. To confirm that PKC ζ is upstream of DHS, we next examined if activation of PKCζ (via its phosphorylation at residue T410) occurred in *Dhpsβ* mice. As shown in Figure 19B, control and Dhpsβ animals on a NCD showed minimal immunostaining for p-PKCζ in islets; by contrast, control animals and *Dhpsβ* animals on a HFD showed apparently equivalent immunostaining intensity for p-PKCζ, suggesting that activation of PKCζ is unimpaired in the absence of *Dhps* (Figure 20C). Collectively, these data suggest that PKCζ activation is unaltered in the absence of DHS activity and its loss impacts hypusine formation.

Next, to clarify the relationship between the DHS/eIF5A pathway and mTOR activity, we utilized the murine β-cell-derived line MIN6. We treated MIN6 cells with 0.5 mM of the free fatty acid (FFA) palmitate as a model *in vitro* to mimic the HFD effects in

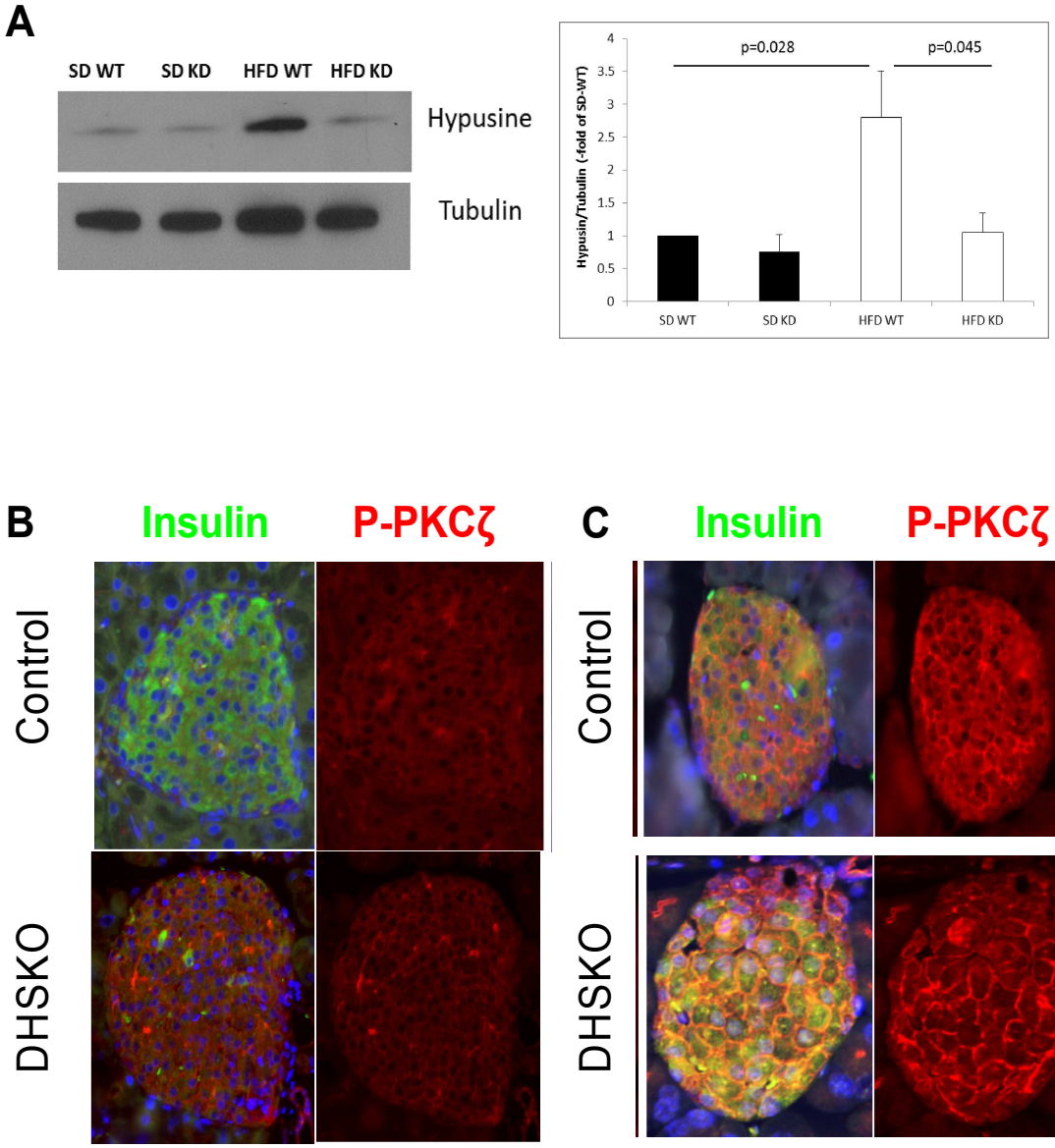


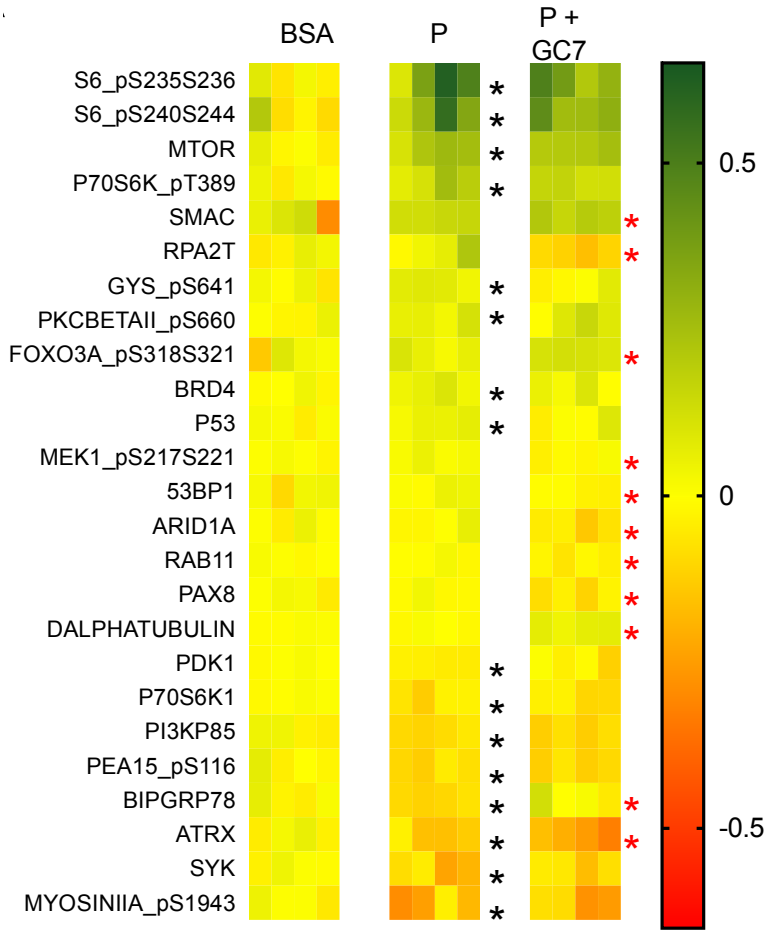
Figure 20: P-PKCζ knockdown affects upstream of DHS activity. PKC KD, DHSΔβ and control mice subjected to 1 weeks of HFD feeding from 10 weeks of age. (**A**) Immunoblot of eIF5A^{Hyp} in isolated islets from PKC knockdown mice fed a standard diet SD or a HFD, quantification of the immunoblots; (**B**) Immunofluorescence of pancreas sections from DHSΔβ mice fed SD for 1 week; (**C**) Immunofluorescence of pancreas sections from DHSΔβ mice fed HFD for 1 week. N=3 to 4 mice total. Data courtesy of the lab of Dr. Adolfo Garcia-Ocaña at the Icahn School of Medicine at Mount Sinai.

mice. Cells were then treated with N1-Guanyl-1,7-diaminoheptane (GC7, a potent and specific inhibitor of DHS (241)) or vehicle, and subjected cell extracts by Reverse Phase Protein Assay (RPPA). As shown in Figure 21 and consistent with prior studies (242), palmitate activated components of the mTOR signaling pathway. Notably, inhibition of DHS with GC7 did not affect activation of this pathway. Further studies are needed to evaluate other molecules that showed downregulation by the GC7 treatment such as the ER stress molecule immunoglobulin heavy–chain binding protein (BIP) and the ATP-dependent helicase X-linked helicase II (ATRX). These findings may suggest that the DHS/eIF5A pathway downstream of PKCζ, but likely in parallel with mTOR signaling.

IV.C. Discussion

Potential approaches to increasing β-cell mass and function during the prediabetic phase of the disease has being gaining an increasing interest in the field (243). The work in this chapter significantly advances our knowledge of the pathways that control β-cell proliferation, and provide a novel contribution on the role of polyamines/DHS/eIF5A in this process. The focus on DHS in this chapter emanated from studies in the literature attributing a role of this enzyme in cellular proliferation/oncogenesis (244–246). Utlizing a novel mouse model not previously described, and subjecting mice to a β-cell mitogenic stimulus, my work in this chapter identified several new findings β-cell proliferation: (a) DHS and its hypusine modification of eIF5A is necessary for the β-cell proliferative response to HFDs in mice, (b) DHS is necessary for the translational engagement of the *Ccnd2* mRNA encoding cyclin-D2 during the stimulus for β-cell proliferation, (c) activity of DHS requires intact PKCζ signaling, but is not may be required for mTOR activation.

The model I employed in this chapter has been utilized by many investigators to demonstrate the capacity of β-cells to proliferate in young mice (212,235,242,247). In my



* p-value < 0.05 Palmitate compared to BSA

* p-value < 0.05 Palmitate + GC7 compared to Palmitate

Figure 21: mTOR pathway unaffected by chemical inhibition of DHS activity. Reverse Protein Profiling Array of total cell lysate of MIN6 treated with Palmitate for 24hr and GC7. N=3-5. *P<0.05 compared to CTL.

study, β-cell mass was measured at two different time points and demonstrated how the block of proliferation as early as 1 week of HFD resulted in a detrimental decrease of β-cell mass by 4 weeks of HFD. The timing of early induction of proliferation in HFD coincides with the hyperglycemia and hyperinsulinemia because both glucose and insulin have been described to induce proliferation and mass increase in rodents in the setting of chronic insulin resistance (248). Continuous hyperglycemia *in vivo* induced proliferation as early as 4 to 7 days after the start of HFD feeding (242). In this study, I confirmed that the levels of hypusination of eIF5A increase in the pancreatic β-cell in response to HFD. This finding explains why we did not see any significant changes following the induction of the DHS knockout. I observed hyperglycemia during both fasting and nonfasting states of the *Dhps*Δ*β* mice after 4 weeks of HFD whereas after 1 week of HFD *Dhps*Δ*β* mice had better response in GTT, indicating that insulin levels are not adequate in the 4 week HFD either by dysfunction or due to a lack of β-cell mass compensation but that DHS deficiency allows for the β-cell to compensate by increasing insulin production and secretion for a short period of time. Because I saw significantly reduced β-cell mass after 4 weeks of HFD in *Dhps*Δ*β* mice, it suggests that DHS and eIF5A^{Hyp} are together responsible for β-cell mass compensation. To determine if the lack of β-cell mass compensation was due to increased β-cell death, I used the DMD assay (as presented in Chapter III) to measure β-cell death in control and *Dhps*Δ*β* mice and did not see differences in β-cell death, strongly suggesting that DHS regulates proliferation during the early compensatory phase. It is important to point out that the study presented in this thesis only evaluated the physiological and molecular effects in a short window, up to 4 weeks of HFD. Recent studies of mice fed a 42% HFD for 16-weeks showed evidence of activation of p53 and reduction in global protein translation (249). This study suggests that p53 plays a role in modulating translation and not transcription of mRNAs during chronic exposure of HFD in the islets.

I explored translational changes during the early stages of β-cell proliferation in response to HFD, focusing on direct regulators of cell cycle progression. Cyclin-D2 is the most important D-cyclin expressed in the β-cell postnatally essential for adult β-cell replication (102). This cyclin forms a complex with and functions as a regulatory subunit of CDK4 (in the case of rodents) and CDK6 (in humans), whose activity is required for cell cycle G1/S transition. It has previously been shown in the literature that there is an increase in cyclin-D2 protein but not mRNA is present during the response to HFD (212). Pancreatectomy studies in mice have shown cyclin-D2 mRNA to be unchanged in the pancreas, but protein levels increased (250). The three D-cyclins contain a phosphorylation site near their carboxyl terminus. It has been shown that mutation of Cyclin-D1 on Thr286 (Threonine286) in mouse embryo fibroblast cell line (NIH3T3) resulted in an increase in proteosomal degradation (251). A more recent study done in a mouse model with cyclin-D2 protein with a mutation on the residue Thr280 to an alanine resulted in a much higher level of protein expression compared to controls despite similar levels of mRNA amounts (250). These studies suggest Cyclin-D2 to be regulated at the protein level, but do not delineate the possible pathways that facilitate an increase on Cyclin-D2 translation under certain conditions despite no changes in mRNA expression. No changes in Cyclin-D2 degradation were observed in response to palmitate treatment in MIN6 studies. I have found that this upregulation of cyclin-D2 protein is dependent on hypusination of eIF5A, since in our *Dhps*Δ*β* mice showed a block of translation leading to significantly reduced protein levels. The results in this study show a potentially novel translational regulatory pathway of DHS influencing cyclin-D2 protein levels in the β-cell under conditions of hyperglycemia and insulin resistance.

PKC-ζ is a relatively novel protein downstream target of phosphatidylinositol 3-kinase-phosphoinositide-dependent kinase-1 (PDK-1). This kinase is critical for

mitogenic signal transduction in many cell types such as fibroblasts, oocytes and glial cells (252,253). Studies in transgenic mouse models of parathyroid hormone-related protein (PTHrP) and hepatocyte growth hormone (HGF) driven by rat insulin promoter (RIP) showed that activation of PKC-ζ resulted in β-cell proliferation (239). Recent studies of KD- PKCζ or by disruption of PKCζ in mouse β-cells resulted in inhibition of compensatory proliferation under acute hyperglycemia/hyperinsulinemia (254). The mammalian target of rapamycin complex1 (mTORC1) is a protein complex that functions as a nutrient/energy sensor controlling protein synthesis. One of its most studied roles is its activation of protein translation to facilitate cell growth and proliferation. mTOR is activated via the serine/threonine kinase Akt pathway activation. It has been suggested in the literature that PKCζ activates early compensation independent of Akt mediated mTOR activation (254). To date, little is known about the regulation of DHS activity. However, based on our data presented here, PKCζ is upstream of hypusination of eIF5A as we showed using kinase dead PKCζ mice a significant reduction of eIF5A^{HYP} when fed HFD compared to controls. However, there was no difference in phosphorylated PKCζ in our DHSΔβ mice compared to controls, confirming our initial assessment of PKCζ being upstream of DHS activation.

Until now, the intracellular pathway of PI3K/Akt/mTOR has been the predominant pathway linking cellular quiescence, proliferation, and longevity of the β-cell. In this chapter, I provide the first evidence that the DHS/eIF5A pathway functions in a parallel fashion to mTORC1, but like mTORC1, lies downstream of PKCζ. The precise link between PKC ζ and hypusine formation still remain unclear. For example, does PKCζ directly regulate ODC and/or DHS, thereby regulating hypusine formation? Or perhaps PKCζ controls another factor (kinase, acetylase, etc.) that subsequently regulates ODC or DHS (Figure 22)?. These and other questions must await further study. Overall, these

studies suggest PKCζ and the polyamine pathway to be parallel in their ability activate cell growth and simultaneously influence activation of DHS and therefore eIF5A activity.

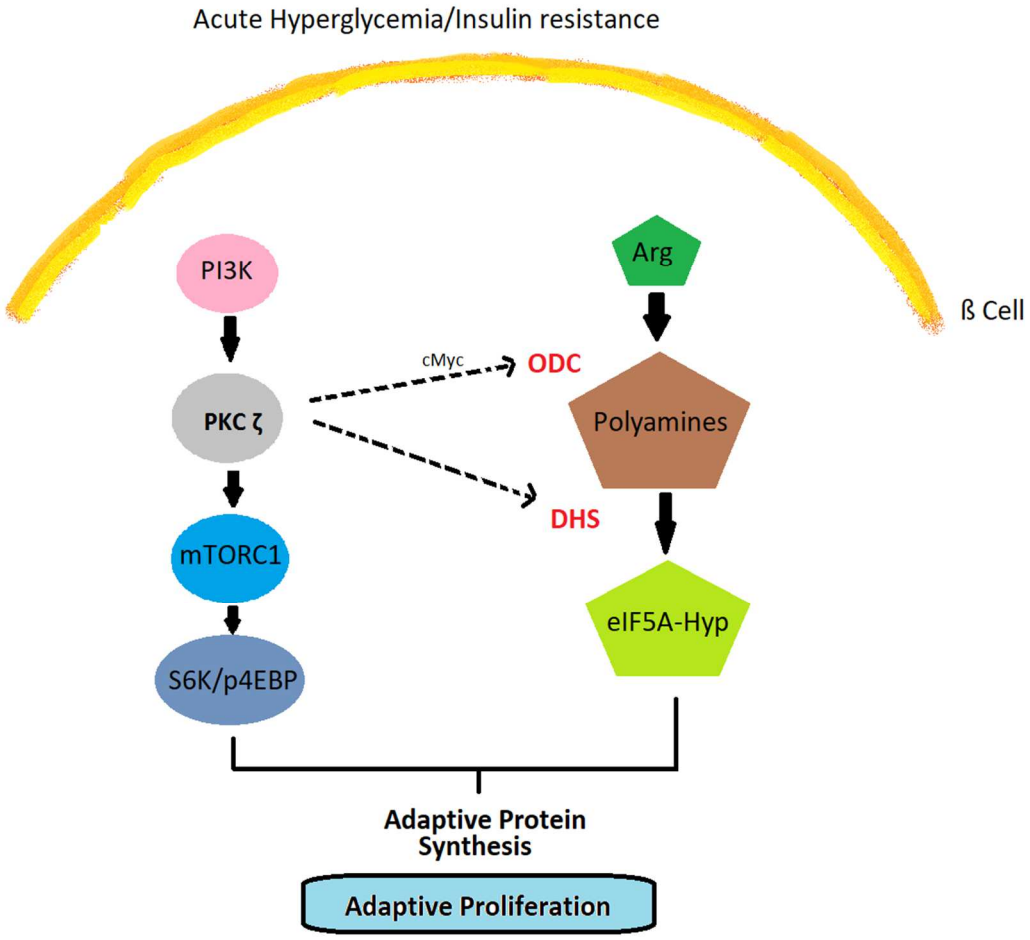


Figure 22: Novel pathway of early compensatory proliferation in the pancreatic β-cell. In the presence of hyperglycemia and insulin resistance PI3K and the polyamine pathway are simultaneously activated. PI3K leads to activation of PKCζ which in turn activates mTORC1 to regulate protein synthesis for proliferation. PKCζ also facilitates the activation of DHS for hypusination of eIF5A resulting in an increase in Cyclin-D2 protein translation leading to compensatory β-cell proliferation.

Chapter V

Conclusions and Future Directions

The research presented in this dissertation provides novel insight into the molecular pathways by which the β-cell mass compensates and decompensates—that is, responds in size and functional capacity—to changing metabolic demands. Generally, these responses include (1) an adaptive proliferative response to a nutritional challenge (Chapter IV) and (2) a maladaptive response in which β-cells are triggered to senesce if the compensatory responses are insufficient to restore euglycemia and re-establish equilibrium (Chapter III). The mechanisms of adaptive compensation are critical to maintain an appropriate range of serum glucose levels in organisms that are exposed to an environment of "overnutrition,"—that is when available energy exceeds the organismal needs for normal growth, development, and metabolism—as might be expected in the setting of obesity. This final chapter will summarize the major accomplishments of this dissertation study and place them into both the historical and contemporary contexts provided in the introductory chapter. Importantly, this chapter will also consider the possible future research questions that stem from the current research.

V.A. The adaptive response to overnutrition: β-cell replication

The seminal studies of Ogilvie (255) and Butler (44) established the associations between islet size, obesity, and diabetes. The activation of β-cell proliferation in response to overnutrition is not only crucial to maintain euglycemia at the early stages of overnutrition, but becomes even more important at later stages with the onset of insulin resistance. As previously described, the mechanisms regulating postnatal β-cell proliferation are complex, comprising multiple molecular pathways that are highly

interdependent (for example, see Figure 3). The longstanding interest in understanding these mechanisms has been fueled by their tantalizing potential to provide novel therapeutic targets that could potentiate survival and function of the β-cell. Previous work in our lab has demonstrated that pathophysiological glucolipotoxicity, thought to be perhaps the instigator of postnatal proliferation, and β-cell mass loss by apoptosis despite intact signaling processes (242,249). The data in this dissertation suggest that glucolipotoxicity promotes postnatal proliferation early on via DHS activation during HFD exposure. The studies I have presented in this dissertation provide evidence not only that the early proliferation response to HFD seen in mice is ultimately essential to prevent diabetes development, but also that DHS and its subordinate eIF5A are involved in this late postnatal increase in proliferation. Moreover, DHS and eIF5A facilitate the translation of specific transcripts, including crucial cell cycle progression molecules like cyclin-D2. Whereas a recent study in yeast suggested that eIF5A (and its bacterial homolog, EFP) is required for translational facilitation of stretches of nascent polypeptides containing poly-proline stretches of as few as two consecutive proline residues (226,256), this finding has not been confirmed in mammalian cells, whose more complex proteome contains a plethora of poly-proline-containing proteins (257). In the case of cyclin-D2, poly-proline stretches do not exist, and therefore the potential that eIF5A has more complex roles in mammalian biology is very likely. Nevertheless, the selective nature of eIF5A with respect to transcript recognition raises the need to address its mechanism using approaches such as ribosome footprinting.

Following the initial observations of nutrition-mediated β-cell proliferation in postnatal rodents (212,235,258), several subsequent reports hypothesized that this response could also be occurring in humans, and that its manipulation could be the key to therapies for diabetes (44). However, clinical findings to support this response *in vivo* are lacking for multiple reasons. These include the inability to study subjects

longitudinally, since there are no reliable non-invasive techniques that currently can be applied in humans. Thus, only human pancreatic tissues routinely available for study are recovered from cadaveric sources. The challenges associated with working with this tissue source significantly limits data collection and interpretation. One possibility to overcome the limitations encountered in studying human β-cell dysfunction is by the development of a non-invasive assessment of functional β-cell mass via ultrasound or magnetic resonance imaging (MRI) but without the risk of high concentrations of radioactivity exposure. Blood tests have been found to be helpful in diagnosing diabetes but not preventing it, therefore an assessment that would allow the longitudinal study of the human islets could provide massive advantages in the field. However, the recent development of a non-invasive β-cell biomarker measuring its viability (as explained in Chapter III), could shed light into understanding β-cell mass changes in humans over time. One could also argue that this assay could be measuring actual stress levels instead of death or failure to proliferate resulting in the release the β-cell DNA as an autocrine signal.

Previous studies in mice have suggested that compensatory proliferation occurs very early in the development of diabetes in response to overnutrition, even prior to insulin resistance (212,235,242). In Chapter IV of this dissertation, the process of β-cell compensation in the presence of elevated glucose/FFAs was investigated. Specifically, since hypusinated eIF5A has been shown to play an important role in cell cycle progression in other cell types (246), we studied the roles of hypusinated eIF5A and DHS during proliferative compensation of β-cell mass. This was a novel direction, as previously all roles identified for DHS and eIF5A in the pancreatic islet have focused on the inflammatory responses in T1D and T2D mouse models with only pharmacologic manipulation (144,167,225,259). Regardless, an emerging interest in understanding how

the β-cell undergoes proliferation in response to increasing insulin demand represents a need in the field to be elucidated.

Studies measuring β-cell replication have shown that proliferation in adults is diminished with age (260). In those studies, it was suggested that younger β-cells can re-enter the cell cycle immediately after mitosis; however, the quiescent interphase period is lengthened with age and shortened by elevated metabolic demands. We determined that DHS deletion in mouse β-cells did not cause any detrimental effects under basal conditions, at least over a short time span. The evidence for this conclusion was based on the comparison of *Dhps*Δ*β* mice and controls, which showed no difference in any of the physiological or molecular characteristics examined, including: enhanced sensitivity to glucose tolerance at 1 week of HFD, cell morphology, and insulin/glucagon expression. Moreover, injury studies have further supported the age-related loss of proliferative capacity. For instance, β-cell proliferation following partial pancreatectomy, and treatment with low-dose STZ and a glucagon-like peptide 1 agonist (261) showed that proliferation was stimulated in young mice but significantly diminished in old mice (261). In these studies, it was found that the adaptive compensatory proliferation that the β-cell can undergo under certain stimuli was severely restricted with advanced age in mice. These experiments suggest that middle-aged mice are largely post-mitotic and young mice may not be a good model for studying regenerative capacity of the β-cells. As such, future studies with regard to replication activation could be performed to identify what molecular pathways are involved and therefore induction of β-cell replication could be performed to specifically determine if increases on a particular molecule facilitates or inhibits further replication. Of particular interest is whether other tissue growth factors may stimulate endogenous regeneration of β-cell mass in patients of different ages with newly diagnosed or long-standing diabetes (262).

Of clinical importance is the improved understanding of signals that regulate growth and maintenance of the β-cells. Many advances have been made involving molecular determinants that allow cell cycle progression from the G_1 phase of the cell cycle. In this context, cyclin-D2 contributes to β-cell replication. A potential challenge to translating this finding to humans is that the molecular triggers for β-cell replication in mice and humans are not the same. It has been shown, however, that β-cell replication indeed is the primary mechanism of growth during infancy and adolescence in humans (91). Other studies have reported that obesity and aging increase the replicative capacity of the β-cell by ~50% and that β-cell mass is actually preserved in human aging (263). Importantly, however, is that the data on cell cycle regulation in β-cell replication is mostly from rodent model studies, and whether these findings can be extended to human β-cells is still unknown. Together, my results strongly suggest future studies to more precisely measure the turnover rate of the human β-cell and whether this can be modulated, as in mice, to meet the need for more insulin via β-cell replication.

Of importance for future study is the fact that there is another factor that may affect the β-cells capacity to overcome stimuli via replication, that is the possibility of the cells not being fully differentiated. Studies interrogating β-cell proliferation with adaptive β-cell function have found that there are two processes of β-cell dysfunction, death and dedifferentiation (264). In fact, studies done in humans scoring dedifferentiated β-cells showed that β-cells accounted for >30% dedifferentiation in T2D cases compared to controls (265). An important direction for future research will be to generate a mouse model were β-cell dedifferentiation can be triggered into other endocrine cells, mimicking the findings in humans with T2D.

In Chapter IV, mechanisms of action to activate DHS and therefore adaptive proliferation in the β-cell was examined *in vivo* in collaboration with Dr. Adolfo Garcia-Ocaña's lab at Mt. Sinai NY. Our results suggested that DHS activity lies downstream of

PKCζ, but in parallel with mTORC1 (Figure 21). This finding serves to link the previously-studied mTOR pathway to DHS/eIF5A, and therefore opens new possibilities regarding the manipulation of β cell replication. For example, could limitations in β-cell replication in aging β-cells be a result not of activation of mTORC1, but rather some limitation linking growth factor signaling to DHS/eIF5A?

V.B. The maladaptive response to overnutrition: β-cell death

To probe the relationship between excess nutrition and β-cell death, the studies described in Chapter III investigated the capacity of a non-invasive biomarker to detect β-cell death *in vivo* in mouse models. Specifically, these data suggest that differential methylation of the preproinsulin gene can be utilized as a potential technique to diagnose β-cell viability. Other studies similarly designed by other groups may complement our approach to confirm the diagnosis (266). In this regard, I hypothesize that if applied to humans, the detection of different levels of preproinsulin DNA in the circulating blood could direct personalized therapies that could prevent or alleviate the development of diabetes.

In our studies, consistent with other reports, exposure of C57BL/6J mice to high fat diet (HFD) was sufficient to induce hyperglycemia. Moreover, the BL/6J mice gained a significant amount of weight in conjunction with the development of hyperglycemia. This was in accord with previous studies that showed an increase in the rate of diabetes development in BL/6J mice (247). Additionally, the β-cells of the BL6 mice showed to have the capability to sustain more environmental changes. This could be due to their different genetic backgrounds, as it has been published that other strains are a more heterogenous genetic strain than BL/6J mice (247). Nevertheless, the observed effects in the BL/6J mouse strain were markedly expected suggesting that β-cells undergo

death in a wave pattern, and death triggers mechanisms that result in temporarily controlling hyperglycemia for a number of weeks until multiple death patterns occur and the resurgence to health no longer happens.Our data presented in Chapter III suggest that because of its sensitivity, this ddPCR/biomarker technology would be invaluable in screening for diabetes at an earlier point in its natural history, before extensive β-cell loss has occurred. Diabetes is typically diagnosed by a failed oral glucose tolerance test that shows fasting blood glucose over 100 mg/dL and 1hr postpandrial blood glucose of 140 mg/dL or more. Obviously, this method of diagnosis increases the rate for macro- and micro-vascular complications in the individual. This early detection in turn might facilitate the implementation of more efficacious interventions that could stop or reverse its course. Some examples of possible interventions to aid with diabetes currently used are exogenous insulin, glucose level controlling medications (Metformin), regimen of diet and exercise. However, these interventions could have better outcomes in preventing future complications in diabetic patients if there was early diagnosis. By early intervention, the disease could possibly be prevented without any pharmacological intervention.

Indeed, our study and others have contributed in developing this technology that could help detect early signs of β cell distress and death and give clinicians a window for treatment that could better the outcomes and/or completely prevent the further development of the disease. In this regard, similar technology has been utilized in other fields with significant success. One example is prenatal cell-free DNA screening which is a widely used method to screen for chromosomal abnormalities in a developing fetus. First reports of the presence of fetal cells in maternal blood was first explained in 1969, were the possibility of isolating cells to identify possible genetic abnormalities was then born (267–269).

V.C. Stress-induced plasticity of β-cells: a fate better than death?

In these studies, we assessed changes in β-cell mass via proliferation and death which may be directly attributed to increased metabolic demands. Similarly, many β-cell injury models have shown that β-cells are capable of recovering from massive insults via self-duplication. However, other studies have shown that β-cell mass may respond to metabolic stresses through changes in cell fate—either conversion of non-beta cells into insulin-expressing beta cells, or de-differentiation to non-insulin expressing cell types. For instance, alpha cells may "trans-differentiate" into β-cells in order to compensate for the lost functional beta cell mass (270). In contrast, the transcriptional regulator FoxO1 is required to maintain β-cell fate under stress (264). It is possible that this parallels the studies in this thesis, as their findings of dedifferentiation of β-cells in the FoxO1-deficient β-cell mouse model that resulted in an elevated number of α-cells seems very similar to findings in which the pancreatic morphology of *Dhps*Δ*β* mice after an extended exposure to HFD. The increase in glucagon-expressing cells seem to be associated with DHS deficiency in the particular diet regimen. Nevertheless, induced compensatory β-cell proliferation seems to be mainly guided by self-replication more than any other mechanism. Studies evaluating cyclin-D2's role in β-cell proliferation also showed similar results in an increase in glucagon expressing cells after an extended period of time. Alternatively, our findings may be the result of cyclin-D2 protein deficiency rather than a direct effect of DHS deficiency. Although it should not be disregarded in this study that other mechanisms are contributing to the resurgence of β-cell mass growth after or during an environmental insult of either acute or of chronic nature.

A future direction based on my research would be to establish how exactly the hypusinated eIF5A affects protein synthesis of cyclin-D2. To date, preliminary findings of RNA immunoprecipitation studies have shown that cyclin-D2 mRNA may be bound

directly to hypusinated eIF5A where its translation is then facilitated. In fact, levels of *Ccnd2* message did not change throughout our studies, suggesting that indeed translation is the main mechanism responsible for controlling proliferation postnatally. In contrast, deletion of cyclin-D2 in the pancreatic β-cell results in similar findings as with the deletion of DHS (205). These findings thus suggest a need in the field to identify precisely which mitogenic signals and activated pathways are responsible for the increase in DHS activity. Yet the finding that DHS and by consequence, hypusinated eIF5A, are responsible for proliferative response is not one that is entirely surprising as the combination has been linked to proliferation in other cell types and diseases (271). Future studies are thus warranted that will examine in more detail precisely how DHS aids in early compensation of the β-cell and when does this positive outcome switches into a detrimental consequence to the β-cell.

The observation in Chapter III that hyperglycemia follows spikes of β-cell death is a phenomenon that even though somewhat discussed in literature, it has never been presented in this level of detail. An initial hypothesis was that perhaps β-cell death would be seen once followed by hyperglycemia and that then followed by the development of diabetes. Yet studies presented in Chapter III refute this hypothesis and rather suggest that waves of β-cell death may occur multiple times throughout a chronic exposure to HFD in mice before frank diabetes develops. Future research could be directed at determining whether this particular aspect of β-cell function in the mouse HFD model has similarities to humans and how intact is β-cell function following the waves of β-cell loss—though this will require the development of better non-invasive techniques of monitoring beta cell mass. One testable hypothesis is that β-cell death is likely to be triggered as they succumb to exhaustion while simultaneously enhancing insulin secretion and self-duplication to ensure that glucose homeostasis is preserved.

Alternately, the rise in β-cell death at certain points during the HFD protocol could be explained by the progressive activation of other stress response pathways, such as those involving NF-κB. This in turn could result in the additional production of reactive oxygen species and the depletion of ER calcium (272), which together would trigger cell death. Indeed, our data suggest that distinct therapeutic approaches may be required to promote β-cell survival in T1D and T2D. Yet these studies suggest that these processes end up activating a similar end result through NF-κB resulting in ER stress provoking a stress response that could give signs of approaches to reduce the effects of β-cell death in both instances. Importantly, stress responses such as the unfolded protein response, which block translation are adaptive in the short term, and serve to maintain the overall functional capacity of the system. However, the question remains whether it is possible to appropriately control the switch between maintaining β-cell mass and maintaining β-cell function.

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Curriculum Vitae

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Education

Doctor of Philosophy, Biochemistry and Molecular Biology — *2017*
Indiana University, Indianapolis, IN

Bachelor of Arts, Biology — *2011*
Indiana University Purdue University Indianapolis, *Indianapolis, IN*

Research Training

Indiana University, Indianapolis, IN — *2013-2017*
Raghu G. Mirmira, MD, PhD
Position: Doctoral Student
Project: Study the regulation of the pancreatic beta cell inflammation by deoxyhypusine synthase and eukaryotic translation initiation factor 5A, and their implications in the diabetic milieu.

Indiana University, Indianapolis, IN — *2012-2013*
Simon J. Conway, PhD
Position: Doctoral Rotating Student
Project: Study genetic manipulations in a mouse model that lead to heart failure in utero, and implications that rescue such defects.

Indiana University, Indianapolis, IN — *2010*
Simon J. Conway, PhD
Position: T-32 NIH Summer Fellowship
Project: Study on the spatiotemporal study of monocytes and macrophages in the embryonic heart. Examine monocytes and macrophage spatiotemporal expression during embryo development in order to examine their role and phagocytic involvement during congenital heart defects.

Indiana University, Indianapolis, IN — *2009*
Lei Wei, PhD
Position: T-35 NIH Summer Fellowship
Project: Design and assemble a DNA construct for a transgenic mouse model where the ROCK I gene had its kinase domain inactivated in cardiomyocytes.

Fellowships and Awards

2007, 2010, 2011	Dean's List High Honors Indiana University Purdue University Indianapolis, IN
2010,2011	NIH T-32, Summer Training Fellowship Indiana University School of Medicine, IN
2009	NIH T-35, Summer Training Fellowship Indiana University School of Medicine, IN
2012-2014	Southern Regional Education Board Scholar Award, IUPUI, (supports student attending Institute Conference for 3 years and mentor attending 2 years to facilitate networking, produce more minority PhDs and encourage people of color to seek faculty positions)
2014	Indiana Clinical and Translational Sciences Institute (CTSI) predoctoral trainee Indiana University School of Medicine, IN This award was forfeited to accept the APS fellowship award
2014-2015	American Physiology Society (APS) Porter Physiology Development Fellow Indiana University School of Medicine, IN
2015	Center for Diabetes and Metabolic Diseases 1st Annual Symposium Poster Award Indiana University School of Medicine, IN
2015-2017	Diabetes and Obesity Research Training Grant (T-32) Indiana University School of Medicine, IN
2016	Annual Pediatric Scholars Day Presentation Winner Riley Hospital for Children, Indianapolis, IN

Publications

Neeb Z, Lajiness JD, Bolanis E and Conway SJ. (2013) Cardiac outflow tract anomalies. WIREs Dev. Biol., doi: 10.1002/wdev.98

Ahlfeld SK, Gao Y, Wang J, Horgusluoglu E, Bolanis E, Clapp DW, and Conway SJ. (2013) Periostin down-regulation is an early marker of inhibited neonatal murine lung alveolar septation. Birth Defects Res A Clin Mol Teratol. 2013 Jun;97(6):373-85. doi: 10.1002/bdra.23149

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Lockett AD, Brown M, Santos-Falcon N, Rush N, Oueni H, Bolanis E, Oberle A, Petrusca D, Serban K, Schweitzer K, Presson R, Campos M and Petrache I. (2014). A1AT Endothelial Trafficking is Blocked by Smoke. PLoS One. 2014 Apr 17;9(4):e93979. doi: 10.1371/journal.pone.0093979

Tersey SA, Bolanis E, Holman TR, Maloney DJ, Nadler JL, and Mirmira RG. (2015). 12-Lipoxygenase and Islet β cell Dysfunction in Diabetes. Mol Endocrinol. 2015 Mar 24:me20151041. doi: 10.1210/me.2015-1041

Tersey SA, Levasseur E, Meah F, Syed F, Sims EK, Farb T, Benninger K, Nelson J, Shaw J, Bokvist K, Evans-Molina C, Mather KJ, Arslanian S, and Mirmira RG. (2017) Cell-Free DNA Biomarkers of of β-Cell Death in Obesity, Glucose Intolerance and Type 2 Diabetes. *In preparation*

Presentations and Abstracts

Esther Levasseur, Sarah A. Tersey, Emily Anderson-Baucum, Bernhard Maier, Raghavendra G. Mirmira. “Posttranslational modification of the factor eIF5A is Required for the Adaptive Response of the Islet Beta Cell During Insulin Resistance”. Center for Diabetes & Metabolic Diseases 3rd Annual Diabetes Symposium, Indianapolis, IN. August, 2017

Esther Bolanis, Sarah A. Tersey, Emily Anderson-Baucum, Bernhard Maier, Raghavendra G. Mirmira. “Posttranslational modification of the factor eIF5A is Required for the Adaptive Response of the Islet Beta Cell During Insulin Resistance”. Midwest Islet Club (MIC) 9th conference, Indianapolis, IN. May, 2016

Esther Bolanis, Sarah A. Tersey, Emily Anderson-Baucum, Bernhard Maier, Raghavendra G. Mirmira. “Posttranslational modification of the factor eIF5A is Required for the Adaptive Response of the Islet Beta Cell During Insulin Resistance”. Annual Pediatric Scholars Day, Indianapolis, IN. May, 2016

Esther Bolanis, Sarah A. Tersey, Emily Anderson-Baucum, Bernhard Maier, Raghavendra G. Mirmira. “Posttranslational modification of the factor eIF5A is Required for the Adaptive Response of the Islet Beta Cell During Insulin Resistance”. Islet Biology Keystone Symposia, Keystone, CO. March, 2016

Esther M. Bolanis, Sarah A. Tersey, Raghavendra G. Mirmira. “Hypusination of eIF5A is required for the proliferative compensation of the islet beta cell during insuling resistance”. Center for Diabetes and Metabolic Diseases (CDMD) 1st Annual Symposium, Indianapolis, IN. August, 2015

Esther M. Bolanis, Sarah A. Tersey, Marisa Fisher, Farah Meah, Thomas B. Farb, Krister Bokvist, Kieren J. Mather, Raghavendra G. Mirmira. “β cell death occurs transiently prior to the development of obesity induced glucose intolerance”, American Diabetes Association (ADA) 75th conference, Boston, MA. June, 2015

Esther M. Bolanis, Sarah A. Tersey, Marisa Fisher, Farah Meah, Thomas B. Farb, Krister Bokvist, Kieren J. Mather, Raghavendra G. Mirmira. “Beta Cell Death Occurs Transiently Prior and Concurrently to the Development of Obesity Induced Glucose Intolerance”, Midwest Islet Club (MIC) 8th conference, Chicago, IL. May, 2015

Angelia D. Lockett, Houssam Oueini , Esther Bolanis , Irina Petrache. Pulmonary Endothelial Cell Transcytosis of Alpha-1 Antitrypsin. American Thoracic Society International Meeting Philadelphia, PA , May, 2013

Esther M. Bolanis, Shawn Ahlfeld, and Simon J Conway. “Do Macrophages play a Primary or Secondary role in BPD Pathogenesis?”, poster symposium at IUPUI, July 2011, and poster symposium for Molecular and Medical Genetics department, September, 2011

Esther M. Bolanis, Shawn Ahlfeld, and Simon J Conway. Do Macrophages play a primary or secondary role in BPD pathogenesis? Medical and Molecular Genetics Poster Symposium. Indianapolis, IN. September, 2011

Esther M. Bolanis, Shawn Ahlfeld, and Simon J Conway. Do Macrophages play a primary or secondary role in BPD pathogenesis? SROP Symposium. IUPUI, Indianapolis, IN. July, 2010.

Esther M. Bolanis, Jianjian Shi, and Lei Wei. “Role of Mutant Kinase Death of Rho-associated coiled-coil Kinase 1 (ROCK 1) in mouse cardiomyocyte apoptosis”, poster symposium at IUPUI and University of Michigan, July, 2009

Esther M. Bolanis, Jianjian Shi, and Lei Wei. Role of Mutant Kinase Death of Rho-associated coiled-coil kinase 1 (ROCK1) in mouse cardiomyocyte apoptosis. SROP Symposium. University of Michigan, Ann Arbor, MI. July 25-26, 2009

Volunteer Experience

2012-2017 IUPUI's IBMG Campus Visit Student Volunteer
Assisted multiple Ph.D applicants to assist during the interview weekends by the following actions: answered questions about the program, gave insight on educational resources, and conversed about tips for the Ph.D application process.

2014-2017 SACNAS recruiting member
Assist in establishing and recruiting members for the new chapter in Indiana University Purdue University in Indianapolis (IUPUI) campus.

2015-2016 Biochemistry Data Club
Assisted in organizing the Biochemistry Data Club during the academic year 2015-2016, scheduling speakers throughout the semester and keeping other graduate students engaged in activities provided by the department.

Organization Affiliations

- Member of Golden Key International Honour Society – IUPUI Chapter
- SREB scholar -Southern Regional Education Board Scholar Award, IUPUI, 2012-2014 (supports student attending Institute Conference for 3 years and mentor attending 2 years to facilitate networking, produce more minority PhDs and encourage people of color to seek faculty positions)
- AAAS member since 2012
- The Underrepresented Professional and Graduate Student Organization (UPnGO) – IUPUI
- American Physiology Society (APS) member since 2014
- Society for Advancement of Hispanics/Chicanos and Native Americans in Science (SACNAS) – IUPUI chapter since 2014